AWARD NUMBER: W81XWH-14-1-0146

TITLE: Comprehensive Evaluation of Altered Systemic Metabolism and Pancreatic Cancer Risk

PRINCIPAL INVESTIGATOR: Dr. Brian Wolpin

CONTRACTING ORGANIZATION: DANA-FARBER CANCER INSTITUTE
Boston, MA 02115

REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	30 Sept 2015 – 29 Sept 2016

4. TITLE AND SUBTITLE
Comprehensive Evaluation of Altered Systemic Metabolism and Pancreatic Cancer Risk

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0146

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Dr. Brian Wolpin

E-Mail: brian_wolpin@dfci.harvard.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

DANA-FARBER CANCER INSTITUTE, INC.
OFFICE OF GRANTS & CONTRACTS
450 BROOKLINE AVE
BOSTON MA 02215-5418

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

An increased risk of pancreatic cancer and reduced survival is seen among patients with altered systemic metabolism, including obesity, hyperglycemia, and hyperinsulinemia. However, the mechanisms by which altered metabolism may promote pancreatic cancer development and growth remain to be identified. The primary purpose of the funded research is to understand the metabolic alterations that promote development of early pancreatic cancers and impact their proliferation. To explore altered metabolism is patients with pancreatic cancer, we are conducting studies in participants from four large prospective cohorts, where individuals provide extensive data on metabolic phenotypes, such as obesity and diabetes, and banked plasma samples for interrogation. The potential impact of understanding the mechanisms underlying early pancreatic cancer growth is substantial, with an improved ability to: (a) diagnose pancreatic cancer at an earlier stage when cure is possible, (b) formulate preventative recommendations based on an individual's metabolic phenotype, and (c) identify novel treatment strategies that disrupt pancreatic tumor metabolism.

15. SUBJECT TERMS
Metabolism, branched chain amino acids, early detection, pancreatic cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	66	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

Page

INTRODUCTION:

An increased risk of pancreatic cancer and reduced survival is seen among patients with altered systemic metabolism, including obesity, hyperglycemia, and hyperinsulinemia. However, the mechanisms by which altered metabolism may promote pancreatic cancer development and growth remain to be identified. The primary purpose of the funded research is to understand the metabolic alterations that promote development of early pancreatic cancers and impact their proliferation. To explore altered metabolism is patients with pancreatic cancer, we are conducting studies in participants from four large prospective cohorts, where individuals provide extensive data on metabolic phenotypes, such as obesity and diabetes, and banked plasma samples for interrogation. The potential impact of understanding the mechanisms underlying early pancreatic cancer growth is substantial, with an improved ability to: (a) diagnose pancreatic cancer at an earlier stage when cure is possible, (b) formulate preventative recommendations based on an individual's metabolic phenotype, and (c) identify novel treatment strategies that disrupt pancreatic tumor metabolism.

KEYWORDS:

Pancreatic cancer; Metabolism; Early detection

ACCOMPLISHMENTS:

What were the major goals of the project?

Four major tasks were described in the approved Statement of Work.

Major Task 1: For all three Specific Aims, local IRB and HRPO approval will be obtained to use the previously collected plasma samples from subjects in four large prospective cohorts.

Major Task 1 included local IRB and HRPO approvals to be completed in the first 5 months of the award to allow evaluation of metabolism in human subjects. This task was completed in the described timeframe.

Major Task 2: Identify appropriate matched case-control set from four cohorts and send plasma samples to the Broad Institute at MIT and Harvard University.

Major Task 2 included generation of a human pancreatic cancer case-control population and aliquotting and shipment of plasma samples for metabolite profiling to the Broad Institute of MIT and Harvard University in months 6-9 of the award. This task was completed in the described timeframe.

Major Task 3: Generate and analyze metabolomics dataset.

Major Task 3 included the analysis by LC-MS of plasma samples for circulating metabolites (months 10-15), quality control of the metabolite data (months 16-17), and analysis of the metabolites related to development of early pancreatic cancer (months 18-24). Two LC-MS platforms were proposed for this work. One method had been completed in the described timeframe. Work to profile metabolites using the second method is ongoing, as described in the previously submitted no cost extension request.

Major Task 4: Generate and analyze metabolites related to branched chain amino acid (BCAA) metabolism.

Major Task 4 included analyses of branched chain amino acid (BCAA) catabolic products (months 10-15), quality control of data (months 16-17), and analysis of BCAA catabolic products with pancreatic cancer development (months 18-23). Evaluation of BCAAs has been completed in the described timeframe. Some of the BCAA catabolic products are included on the second LC-MS platform and their analysis is ongoing.

What was accomplished under these goals?

1) Major activities: The primary activities conducted during the second 12 months of this award were related to analysis of circulating plasma metabolites and their association with pancreatic cancer development and progression.

2) Specific objectives: The primary objective in the second year of this award was to generate and analyze a high-quality, thoroughly-vetted dataset of pancreatic cancer cases and controls with extensive clinical characterization and >4,000 measured plasma metabolites. This dataset forms the basis for defining metabolic determinant of early pancreatic cancer development and progression.

3) Significant results: The important result from the second year of the award is the successful analysis of >2,000 plasma metabolites using our non-targeted LC-MS platforms in 1,500 pancreatic cancer cases and controls, as proposed in our award application.

Using LC-MS Method 1, we identified 2,477 circulating metabolites that could be reliably aligned across the plasma samples from participants in four prospective cohort studies (HPFS, NHS, PHS and WHI). Of these metabolites, 1,228 were excluded because their coefficients of variance were > 25%. Of the remaining metabolites, 32 were excluded because data were missing for >10% of participants. Finally, we assessed stability with processing delay. Samples from HPFS and NHS were shipped overnight on ice. Thus, metabolites not stable during this time period must be excluded from analyses. We performed a pilot study, whereby we simulated these conditions and included the samples in a blinded fashion within the actual study participant samples. Of the metabolites that passed our first two QC metrics, another 199 metabolites were not stable on processing delay and were excluded from the final data set. Thus, after our 3-level QC process, 1,018 metabolites were of sufficient quality to be analyzed in our nested case-control set.

Using conditional logistic regression, we have identified 20 metabolites with FDR P-value<0.1. The raw P-values for these metabolites range from 3.3×10^{-5} to 1.9×10^{-3}. Of these 20 metabolites, four are “knowns”, including isoleucine, leucine, valine, and glycine. We identified the three BCAAs (isoleucine, leucine, and valine) in a previously published study (Mayers et al, Nat Med, 2014). In addition to these 4 known metabolites, we also identified 16 “unknowns”, which are metabolites of unknown composition. We are working to determine the identity of each of these unknowns. Interestingly, seven of the 17 non-BCAA metabolites have low correlation coefficients (rho<0.3) with the BCAAs or previously measured markers of insulin resistance, suggesting novel mechanisms of association. As expected risk estimates for these metabolites do not change when considering known risk factors for PDAC in multivariable-adjusted models, including BCAAs, body-mass index, and circulating markers of insulin resistance.

In addition to single metabolite models, we are currently building multi-metabolite risk models that simultaneously incorporate information from correlated metabolites. The goal of this work is to better stratify the population by PDAC risk, while building a better understanding of the biology underlying disease development.

4) Other achievements: During the second year of this award, we published four further manuscripts related to the proposed work.

In the first manuscript, we examined the association of plasma levels of 25-hydroxyvitamin D (25[OH]D) with patient survival, demonstrating that low levels of 25(OH) are associated with lower survival time. This manuscript was published in *Journal of Clinical Oncology*.

Yuan C, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetselaar LG, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol*. 2016;34(24):2899-905.

Purpose: Although vitamin D inhibits pancreatic cancer proliferation in laboratory models, the association of plasma 25-hydroxyvitamin D (25[OH]D) with patient survival is largely unexplored. **Patients and Methods**: We analyzed survival among 493 patients from five prospective U.S. cohorts diagnosed with pancreatic cancer in 1984-2008. We estimated hazard ratios (HRs) for death by plasma level of 25(OH)D (insufficient, <20 ng/ml; relative insufficiency, 20-<30 ng/ml; sufficient ≥30 ng/ml) using Cox proportional hazards models adjusted for age, cohort, race/ethnicity, smoking, diagnosis year, stage, and blood collection month. We also evaluated 33 tagging single nucleotide polymorphisms (SNPs) in the vitamin D receptor gene (*VDR*), requiring $P<0.002$ (0.05/33) for statistical significance. **Results**: The mean prediagnostic plasma level of 25(OH)D was 24.6 ng/ml, and 165 (33%) patients were vitamin D insufficient. Compared to patients with insufficient levels, multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48-1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49-0.90) for patients with sufficient levels (P-trend=0.01; see Table 2 of attached manuscript). These results were unchanged after further adjustment for body-mass index and history of diabetes (P-trend=0.02; see Table 2 of attached manuscript). The association was strongest among patients with blood collected within 5 years of diagnosis, with a HR of 0.58 (95% CI, 0.35-0.98) comparing patients with sufficient to patients with insufficient 25(OH)D levels (see Table 4 of attached manuscript). No SNP at *VDR* met our corrected significance threshold of $P<0.002$; rs7299460 was mostly strongly associated with survival (HR per minor allele, 0.80; 95% CI, 0.68-0.95; $P=0.01$; see Table A3 of attached manuscript). **Conclusion**: We observed longer overall survival in patients with pancreatic cancer who had sufficient prediagnostic plasma levels of 25(OH)D.

In the second manuscript, we investigated markers of obesity, leptin signaling and pancreatic cancer risk in our nested cases and controls, demonstrating elevated risk for pancreatic cancer among men with high plasma leptin levels. This manuscript was published in *Cancer Research*.

Babic A, Bao Y, Qian ZR, Yuan C, Giovannucci EL, Aschard H, Kraft P, Amundadottir LT, Stolzenberg-Solomon RZ, Morales-Oyarvide V, Ng K, Stampfer MJ, Ogino S, Buring JE, Sesso HD, Gaziano JM, Rifai N, Pollak MN, Anderson ML, Cochrane BB, Luo J, Manson JE, Fuchs CS, Wolpin B. Pancreatic cancer risk associated with prediagnostic plasma levels of leptin and leptin receptor genetic polymorphisms. Cancer Res. 2016 Oct 25. [Epub ahead of print]

Leptin is an adipokine involved in regulating energy balance which has been identified as a potential biological link in development of obesity-associated cancers such as pancreatic cancer. In this prospective, nested case-control study of 470 cases and 1094 controls from five U.S. cohorts, we used conditional logistic regression to evaluate pancreatic cancer risk by prediagnostic plasma leptin, adjusting for race/ethnicity, diabetes, body-mass index, physical activity, plasma C-peptide, adiponectin, and 25-hydroxyvitamin D. Due to known differences in leptin levels by gender, analyses were conducted separately for men and women. We also evaluated associations between 32 tagging single nucleotide polymorphisms (SNPs) in the leptin receptor (LEPR) gene and pancreatic cancer risk. Leptin levels were higher in female versus male control participants (median, 20.8 vs. 6.7μg/mL; P<0.0001). Among men, plasma leptin was positively associated with pancreatic cancer risk, and those in the top quintile had a multivariable-adjusted odds ratio (OR) of 3.02 compared to men in the bottom quintile (95% CI, 1.27-7.16; Ptrend=0.02; see Table 2 of attached manuscript). Among women, circulating leptin was not associated with pancreatic cancer risk (Ptrend=0.21; see Table 2 of attached manuscript). Results were similar across cohorts (Pheterogeneity=0.88 for two male cohorts and 0.35 for three female cohorts; see Figure 2 of attached manuscript). In genetic analyses, rs10493380 in LEPR was associated with increased pancreatic cancer risk among women, with an OR per minor allele of 1.54 (95% CI, 1.18-2.02; multiple hypothesis-corrected P=0.03; see Table 3 of attached manuscript). No SNPs were significantly associated with risk in men. In conclusion, higher prediagnostic levels of plasma leptin were associated with an elevated risk of pancreatic cancer among men, but not among women.

In the third manuscript, we worked with colleagues at Massachusetts Institute of Technology to understand how BCAAs are used by pancreatic tumors. This manuscript was published in *Science*.

Mayers JR, Torrence ME, Danai LV, Papagiannakopoulos T, Davidson SM, Bauer MR, Lau AN, Ji BW, Dixit PD, Hosios AM, Muir A, Chin CR, Freinkman E, Jacks T, Wolpin BM, Vitkup D, Vander Heiden MG. Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. *Science*. 2016;353(6304):1161-5.

Tumor genetics guides patient selection for many new therapies, and cell culture studies have demonstrated that specific mutations can promote metabolic phenotypes. However, whether tissue context defines cancer dependence on specific metabolic pathways is unknown. Kras activation and Trp53 deletion in the pancreas or the lung result in pancreatic ductal adenocarinoma (PDAC) or non-small cell lung carcinoma (NSCLC), respectively, but despite the same initiating events, these tumors use branched-chain amino acids (BCAAs) differently (see Figure 1 of attached manuscript). NSCLC tumors incorporate free BCAAs into tissue protein and use BCAAs as a nitrogen source, whereas PDAC tumors have decreased BCAA uptake. These differences are reflected in expression levels of BCAA catabolic enzymes in both mice and humans (see Figure 3 in the attached manuscript). Loss of Bcat1 and Bcat2, the enzymes responsible for BCAA use, impairs NSCLC tumor formation, but these enzymes are not required for PDAC tumor formation, arguing that tissue of origin is an important determinant of how cancers satisfy their metabolic requirements (see Figure 4 of attached manuscript).

In the fourth manuscript, we performed a study to evaluate factors at the time of blood collection that impact metabolite measurements. These factors are important to consider in large human studies that incorporate metabolite profiling. This manuscript was published in *Cancer Epidemiology Biomarkers & Prevention*.

Townsend MK, Bao Y, Poole EM, Bertrand KA, Kraft P, Wolpin BM, Clish CB, Tworoger SS. Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics. *Cancer Epidemiol Biomarkers Prev*. 2016;25(5):823-9.

BACKGROUND: Many epidemiologic studies are using metabolomics to discover markers of carcinogenesis. However, limited data are available on the influence of pre-analytic blood collection factors on metabolite measurement. **METHODS**: We quantified 166 metabolites in archived plasma from 423 Health Professionals Follow-up Study and Nurses' Health Study participants using liquid chromatography-tandem mass spectrometry (LC-MS). We compared multivariable-adjusted geometric mean metabolite LC-MS peak areas across fasting time, season of blood collection, and time of day of blood collection categories. **RESULTS**: The majority of metabolites (160 of 166 metabolites) had geometric mean peak areas that were within 15% comparing samples donated after fasting 9 to 12 versus ≥13 hours; greater differences were observed in samples donated after fasting ≤4 hours (see Table 3 in attached manuscript). Metabolite peak areas generally were similar across season of blood collection, although levels of certain metabolites (e.g., bile acids and purines/pyrimidines) tended to be different in the summer versus winter months (see Table 4 of the attached manuscript). After adjusting for fasting status, geometric mean peak areas for bile acids and vitamins, but not other metabolites, differed by time of day of blood collection (see Table 5 of the attached manuscript). **CONCLUSION**: Fasting, season of blood collection, and time of day of blood collection were not important sources of variability in measurements of most metabolites in our study. However, considering blood collection variables in the design or analysis of studies may be important for certain specific metabolites, particularly bile acids, purines/pyrimidines, and vitamins. **IMPACT**: These results may be useful for investigators formulating analysis plans for epidemiologic metabolomics studies, including determining which metabolites to a priori exclude from analyses.

What opportunities for training and professional development has the project provided?
During the second year of this award, Dr. Wolpin has met regularly with Dr. Clary Clish at the Broad Institute of MIT and Harvard University and Dr. Peter Kraft of the Harvard School of Public Health. These meeting have entailed detailed review of study design, analysis plans, and the analytic characteristics of the LC-MS platforms. The close proximity of Dana-Faber Cancer Institute, Harvard School of Public Health and Broad Institute enables these regular, in-person meetings. Dr. Wolpin meets regularly with the Designated Collaborator for the award, Dr. Charles Fuchs. Dr. Fuchs' office is adjacent to Dr. Wolpin's allowing for both regularly scheduled meetings and numerous impromptu discussions regarding design and analysis considerations. Furthermore, Dr. Wolpin has a graduate student and instructor in his group that accompany him to these meetings, allowing for further training and professional development opportunities for more junior investigators. Dr. Wolpin continues attend a number of relevant conferences at Dana-Farber and other nearby Harvard institutions. He presents his work at the Channing Laboratory cohort study meetings, obtaining real-time feedback and mentoring from senior faculty. He attends the DFCI Seminars in Oncology series and had the opportunity to present his work at this Institute-wide conference. He also traveled to several conference and delivered talks on his work, including the American Association for Cancer Research (AACR) Integrative Molecular Epidemiology Workshop (Boston, MA), Cold Spring Harbor Laboratory Workshop on Vitamin D and Pancreatic Cancer (Cold Spring Harbor, NY), and NCI Cohort Consortium annual meeting (Rockville, MD). Thus, Dr. Wolpin has had ample opportunities to expand his training and professional development during the first year of the award.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?
During the no-cost extension period of the award, we will complete our metabolite analyses, including individual known and unknown metabolites, BCAA catabolic products, and pathway analysis modules developed at the Broad Institute.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
The initiated work will advance our understanding of how pancreatic cancers use nutrients to promote their growth. This has important applications to early detection of pancreatic cancer and identifying new treatments that cut off the nutrient supply to tumors.

What was the impact on other disciplines?
The work performed within this award will advance the field technically by demonstrating how to pursue large-scale metabolism studies using banked blood samples from thousands of individuals. This has relevance to many disease states, beyond cancer.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
This work has the potential to increase awareness around pancreatic cancer, which is now the third leading cause of cancer-related death in the United States. Furthermore, this work has the potential to highlight the link between obesity, diabetes, and cancer, which has important implications for lifestyle recommendations to the general public.

CHANGES/PROBLEMS:

Changes in approach and reasons for change
Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to Report

Changes that had a significant impact on expenditures
Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to Report

Significant changes in use or care of human subjects
Nothing to Report

Significant changes in use or care of vertebrate animals.
Nothing to Report

Significant changes in use of biohazards and/or select agents
Nothing to Report

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

1. Yuan C, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetselaar LG, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol*. 2016;34(24):2899-905.
2. Babic A, Bao Y, Qian ZR, Yuan C, Giovannucci EL, Aschard H, Kraft P, Amundadottir LT, Stolzenberg-Solomon RZ, Morales-Oyarvide V, Ng K, Stampfer MJ, Ogino S, Buring JE, Sesso HD, Gaziano JM, Rifai N, Pollak MN, Anderson ML, Cochrane BB, Luo J, Manson JE, Fuchs CS, Wolpin BM. Pancreatic cancer risk associated with prediagnostic plasma levels of leptin and leptin receptor genetic polymorphisms. *Cancer Res*. 2016 Oct 25. [Epub ahead of print]
3. Mayers JR, Torrence ME, Danai LV, Papagiannakopoulos T, Davidson SM, Bauer MR, Lau AN, Ji BW, Dixit PD, Hosios AM, Muir A, Chin CR, Freinkman E, Jacks T, Wolpin BM, Vitkup D, Vander Heiden MG. Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. *Science*. 2016;353(6304):1161-5.
4. Townsend MK, Bao Y, Poole EM, Bertrand KA, Kraft P, Wolpin BM, Clish CB, Tworoger SS. Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics. *Cancer Epidemiol Biomarkers Prev*. 2016;25(5):823-9

Books or other non-periodical, one-time publications.
Nothing to Report.

Other publications, conference papers, and presentations.

Local presentations:

2016	Precision Medicine for Pancreatic Adenocarcinoma	Center for Cancer Precision Medicine Tumor Board Conference
Dana-Farber Cancer Institute		
2016	Precision Medicine for Pancreatic Adenocarcinoma	Conference of the Hale Center for Pancreatic Cancer Research
Dana-Farber Cancer Institute		

National presentations:

2015	Epistatic Targeting of Primary and Metastatic Pancreatic Cancer	Invited Speaker
Stand Up to Cancer – Cancer Research UK – Lustgarten		Philadelphia, PA

Foundation Dream Team Finalist Presentation

2015	Research Biopsy Program in Patients with Metastatic Pancreatic Cancer	Invited Speaker
Lustgarten Foundation Scientific Meeting		Cold Spring Harbor, NY
2015	The Need for Both Edges of the Sword: Early Detection and New Treatment Approaches for Pancreatic Cancer Mortality Reduction	Invited Speaker
MD Anderson Cancer Center Ahmad Center Seminar Series		Houston, TX
2016	The Double-edged Sword for Pancreatic Cancer Mortality Reduction: Identifying New Approaches for Early Detection and Treatment	Invited Speaker
Oregon Health & Science University Seminar Series, Brenden-Colson Center for Pancreatic Care		Portland, OR
2016	Altered Systemic Metabolism and Pancreatic Adenocarcinoma	Invited Speaker
University of California at San Francisco, Pancreas Center and Diabetes & Obesity Research Seminar Series		San Francisco, CA
2016	Pancreatic Adenocarcinoma and Altered Host Metabolism	Invited Speaker
American Society of Preventative Oncology 40th Annual Meeting		Columbus, OH
2016	Dana-Farber Pancreatic Cancer Research Tissue Platform	Invited Speaker
Pancreatic Cancer Action Network RAN-2 Grant Meeting		Rochester, NY
2016	Defining Novel Approaches to Early Detection and Treatment of Pancreatic Adenocarcinoma	Invited Speaker
The Ohio State University Comprehensive Cancer Center Seminar Series		Columbus, OH
2016	Defining Novel Approaches to Early Detection and Treatment of Pancreatic Adenocarcinoma	Invited Speaker
University of Michigan Comprehensive Cancer Center Seminar Series		Ann Arbor, MI
2016	Metabolomics and Cancer	Invited Speaker

American Association for Cancer Research (AACR) Integrative Molecular Epidemiology Workshop	Boston, MA

International Invited Presentations

2016	Altered Systemic Metabolism and Early Pancreatic Adenocarcinoma	Invited Speaker
Pezcoller Symposium		Trento, Italy

In the above presentations, metabolism changes due to pancreatic cancer were discussed, including how these changes can inform early detection and prognosis of pancreatic cancer.

Website(s) or other Internet site(s)
Nothing to Report.

Technologies or techniques
Nothing to Report.

Inventions, patent applications, and/or licenses
Nothing to Report.

Other Products
Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?
Brian Wolpin. No change.
Chen Yuan. No change.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
See attached Other Support page.

What other organizations were involved as partners?
Nothing to Report.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Not applicable.

QUAD CHARTS:
Not applicable.

APPENDICES:

Four manuscripts:

1. Yuan C, Qian ZR, Babic A, Morales-Oyarvide V, Rubinson DA, Kraft P, Ng K, Bao Y, Giovannucci EL, Ogino S, Stampfer MJ, Gaziano JM, Sesso HD, Buring JE, Cochrane BB, Chlebowski RT, Snetselaar LG, Manson JE, Fuchs CS, Wolpin BM. Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival. *J Clin Oncol*. 2016;34(24):2899-905.
2. Babic A, Bao Y, Qian ZR, Yuan C, Giovannucci EL, Aschard H, Kraft P, Amundadottir LT, Stolzenberg-Solomon RZ, Morales-Oyarvide V, Ng K, Stampfer MJ, Ogino S, Buring JE, Sesso HD, Gaziano JM, Rifai N, Pollak MN, Anderson ML, Cochrane BB, Luo J, Manson JE, Fuchs CS, Wolpin BM. Pancreatic cancer risk associated with prediagnostic plasma levels of leptin and leptin receptor genetic polymorphisms. *Cancer Res*. 2016 Oct 25. [Epub ahead of print]
3. Mayers JR, Torrence ME, Danai LV, Papagiannakopoulos T, Davidson SM, Bauer MR, Lau AN, Ji BW, Dixit PD, Hosios AM, Muir A, Chin CR, Freinkman E, Jacks T, Wolpin BM, Vitkup D, Vander Heiden MG. Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. *Science*. 2016;353(6304):1161-5.
4. Townsend MK, Bao Y, Poole EM, Bertrand KA, Kraft P, Wolpin BM, Clish CB, Tworoger SS. Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics. *Cancer Epidemiol Biomarkers Prev*. 2016;25(5):823-9

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival

Chen Yuan, Zhi Rong Qian, Ana Babic, Vicente Morales-Oyarvide, Douglas A. Rubinson, Peter Kraft, Kimmie Ng, Ying Bao, Edward L. Giovannucci, Shuji Ogino, Meir J. Stampfer, John Michael Gaziano, Howard D. Sesso, Julie E. Buring, Barbara B. Cochrane, Rowan T. Chlebowski, Linda G. Snetselaar, JoAnn E. Manson, Charles S. Fuchs, and Brian M. Wolpin

Author affiliations appear at the end of this article.

Published online ahead of print at www.jco.org on June 20, 2016.

Supported by National Institutes of Health (NIH), National Cancer Institute (NCI) Grant No. K07-CA148894 (to K.N.); NIH NCI Grant No. R35-CA197735 (to S.O.); the Robert T. and Judith B. Hale Fund for Pancreatic Cancer, Perry S. Levy Fund for Gastrointestinal Cancer Research, Pappas Family Research Fund for Pancreatic Cancer, and NIH NCI Grants No. R01-CA124908 and P50-CA127003 (to C.S.F.); and by Department of Defense Grant No. CA130288, Lustgarten Foundation, the Noble Effort Fund, the Peter R. Leavitt Family Fund, and Promises for Purple (to B.M.W.). The Health Professionals Follow-Up Study is supported by NIH Grant No. UM1 CA167552. The Nurses' Health Study is supported by NIH Grants No. UM1 CA186107, P01 CA87969, and R01 CA49449. The Physicians' Health Study is supported by NIH Grants No. CA 34944, CA 40360, HL 26490, and HL 34595. The Women's Health Initiative program is funded by the NIH through Contracts No. N01WH22110, 24152, 32100-2, 32105-6, 32108-9, 32111-13, 32115, 32118-32119, 32122, 42107-26, 42129-32, and 44221.

Authors' disclosures of potential conflicts of interest are found in the article online at www.jco.org. Author contributions are found at the end of this article.

Corresponding author: Brian M. Wolpin, MD, MPH, Dana-Farber Cancer Institute, 450 Brookline Ave, Boston, MA 02215; e-mail: bwolpin@partners.org.

0732-183X/16/3499-1/$20.00

DOI: 10.1200/JCO.2015.66.3005

ABSTRACT

Purpose
Although vitamin D inhibits pancreatic cancer proliferation in laboratory models, the association of plasma 25-hydroxyvitamin D [25(OH)D] with patient survival is largely unexplored.

Patients and Methods
We analyzed survival among 493 patients from five prospective US cohorts who were diagnosed with pancreatic cancer from 1984 to 2008. We estimated hazard ratios (HRs) for death by plasma level of 25(OH)D (insufficient, < 20 ng/mL; relative insufficiency, 20 to < 30 ng/mL; sufficient ≥ 30 ng/mL) by using Cox proportional hazards regression models adjusted for age, cohort, race and ethnicity, smoking, diagnosis year, stage, and blood collection month. We also evaluated 30 tagging single-nucleotide polymorphisms in the vitamin D receptor gene, requiring $P < .002$ (0.05 divided by 30 genotyped variants) for statistical significance.

Results
Mean prediagnostic plasma level of 25(OH)D was 24.6 ng/mL, and 165 patients (33%) were vitamin D insufficient. Compared with patients with insufficient levels, multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48 to 1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49 to 0.90) for patients with sufficient levels (P trend = .01). These results were unchanged after further adjustment for body mass index and history of diabetes (P trend = .02). The association was strongest among patients with blood collected within 5 years of diagnosis, with an HR of 0.58 (95% CI, 0.35 to 0.98) comparing patients with sufficient to patients with insufficient 25(OH)D levels. No single-nucleotide polymorphism at the vitamin D receptor gene met our corrected significance threshold of $P < .002$; rs7299460 was most strongly associated with survival (HR per minor allele, 0.80; 95% CI, 0.68 to 0.95; $P = .01$).

Conclusion
We observed longer overall survival in patients with pancreatic cancer who had sufficient prediagnostic plasma levels of 25(OH)D.

J Clin Oncol 34. © 2016 by American Society of Clinical Oncology

INTRODUCTION

Pancreatic cancer is the third-leading cause of cancer-related deaths in the United States, and most patients die within 12 months of diagnosis.[1] Other than disease stage at presentation, few patient characteristics or circulating markers have been identified that predict survival time in patients with pancreatic ductal adenocarcinoma.[2]

Vitamin D is a fat-soluble vitamin that can be ingested in foods and is produced endogenously when sunlight triggers synthesis in the skin. Metabolites of vitamin D bind to the vitamin D receptor (VDR) within cells, which regulate transcription of target genes by interacting with vitamin D response elements.[3] In addition to the essential roles of vitamin D in calcium homeostasis and bone health, emerging evidence indicates that vitamin D and its analogs may inhibit cancer development and growth.[3-5] Recent studies in laboratory models of pancreatic cancer have demonstrated the therapeutic potential of vitamin D analogs by engagement of VDR in tumor cells and supportive cells within the tumor stroma.[6-8] Although studies that have evaluated circulating vitamin D and pancreatic cancer incidence have been conflicting,[9,10] few studies have

been conducted to assess the association of vitamin D levels with survival in patients with pancreatic cancer.[11,12]

To assess the potential prognostic effect of vitamin D status, we evaluated the association between prediagnostic plasma 25-hydroxyvitamin D [25(OH)D] and overall survival among patients with pancreatic cancer drawn from five large US prospective cohorts. In addition, we examined survival in relation to common germline genetic variants in *VDR*.

PATIENTS AND METHODS

Study Population

Our study population included patients with pancreatic cancer from five US prospective cohort studies. The Health Professionals Follow-up Study (HPFS) was established in 1986 when 51,529 male health professionals, age 40 to 75 years, responded to a mailed questionnaire. The Nurses' Health Study (NHS) was initiated in 1976 when 121,700 female registered nurses, age 30 to 55 years, responded to a mailed questionnaire. The Physicians' Health Study I (PHS I) is a randomized clinical trial of aspirin and β-carotene that enrolled 22,071 male physicians, age 40 to 84 years, in 1982. After completion of the randomly assigned components, study participants were observed. The Women's Health Initiative (WHI) Observational Study enrolled 93,676 postmenopausal women, age 50 to 79 years, between 1994 and 1998. The Women's Health Study (WHS) is a randomized, clinical trial of low-dose aspirin and vitamin E that enrolled 39,876 female professionals, age ≥ 45 years, between 1992 and 1995. The trial was completed in 2004, and participants were observed. Details of these cohorts have been described previously (HPFS,[13] NHS,[14] PHS,[15] WHI,[16] and WHS[17]). The study was approved by the Human Research Committee at the Brigham and Women's Hospital, and participants provided informed consent.

We included 493 patients with pancreatic adenocarcinoma who were diagnosed through 2008 and who had available plasma and no prior history of cancer, except nonmelanoma skin cancer. Incident cases of pancreatic cancer were identified by self-report or during follow-up of participant deaths. Deaths were ascertained from next-of-kin or the US Postal Service and by searching the National Death Index; this method has been shown to capture > 98% of deaths.[18] Diagnoses were confirmed by review of medical records, death certificates, and/or tumor registry data by study physicians who were blinded to exposure data. Patients with non-adenocarcinoma histology or unclear survival time were excluded.

Individual characteristics and habits were obtained from baseline questionnaires at study enrollment in PHS, WHI, and WHS, and from questionnaires that preceded the date of blood draw in HPFS and NHS. In all cohorts, data were available for age at blood draw, sex, race/ethnicity, weight, height, smoking status, and history of diabetes. Date of diagnosis and pancreatic cancer stage at diagnosis were obtained from medical record review. Cancer stage was classified as local disease amenable to surgical resection; locally advanced disease with extrapancreatic extension rendering it unresectable, but without distant metastases; distant metastatic disease; or unknown.

Plasma Samples

Blood samples were collected from 18,225 male patients in HPFS from 1993 to 1995; 32,826 female patients in NHS from 1989 to 1990; 14,916 male patients in PHS from 1982 to 1984; 93,676 female patients in WHI from 1994 to 1998; and 28,345 female patients in WHS from 1992 to 1995. All blood samples were continuously stored in well-monitored freezers. Details on blood draw procedures, transportation, and storage of plasma samples in these cohorts have been described elsewhere.[19] As described previously, plasma levels of 25(OH)D were assayed in the laboratory of Nader Rifai, MD (Children's Hospital, Boston, MA), by using the 25-Hydroxyvitamin D Enzyme Immunoassay Kit from Immunodiagnostic Systems (Tyne & Wear, United Kingdom) per manufacturer instructions.[9] Mean intra-assay coefficients of variance were ≤ 9% for blinded, replicate, quality control samples.[9]

Single Nucleotide Polymorphism Selection and Genotyping

We selected 36 tagging single nucleotide polymorphisms (SNPs) in the *VDR* gene ± 20 kb by using the tagger algorithm implemented in Haploview, with a cutoff of $r^2 = 0.8$ and a minor allele frequency of ≥ 5% in Whites from the HapMap Project database, and we forced in one previously reported SNP related to prostate cancer risk (rs11574143).[20] Of 397 patients with pancreatic cancer, DNA was extracted from archived buffy coat samples with Qiagen QIAmp (Valencia, CA) and whole-genome amplified with GE Healthcare Genomiphi (Pittsburgh, PA). All genotyping was carried out at the Partners HealthCare Center for Personalized Genetic Medicine by using a custom-designed Illumina Golden Gate genotyping assay (San Diego, CA). Three tagging SNPs were not supported by the Golden Gate platform and so could not be genotyped. Three SNPs deviated from Hardy-Weinberg Equilibrium at $P < .01$ and were excluded. Replicate samples included for quality control (n = 44 sample groups) had mean genotype concordance of 97.8% across the 30 SNPs.

Statistical Analysis

Association of plasma 25(OH)D with overall survival was examined by using Cox proportional hazards regression models to calculate hazard ratios (HRs) and 95% CI. Overall survival time was calculated from the date of cancer diagnosis until the date of death or last follow-up if a participant was still alive. Although optimal levels of 25(OH)D have not been definitively determined, plasma 25(OH)D < 20 ng/mL has been defined as insufficiency, 20 to < 30 ng/mL as relative insufficiency, and ≥ 30 ng/mL as sufficient[21,22]; therefore, we investigated patient survival by these categories of plasma 25(OH)D. To compare more extreme levels of 25(OH)D in secondary analyses, we assessed the association between quintiles of 25(OH)D and survival. We examined HRs for each gender-specific cohort individually and computed a summary HR by using the DerSimonian and Laird random effects model.[23] Heterogeneity was tested by using Cochran's Q statistic.[24] Two-sided tests for trend were calculated by entering log-transformed 25(OH)D as a continuous variable into Cox proportional hazards regression models, given that 25(OH)D was not normally distributed. In multivariable models, we adjusted for potential confounders, including age at diagnosis, race and ethnicity, smoking status, year of diagnosis, cancer stage, month of blood collection, and time between blood collection and cancer diagnosis. Subsequently, we adjusted for body mass index (BMI) and history of diabetes, which were prognostic factors identified in prior studies of these populations.[25,26] The proportionality of hazards assumption was satisfied by evaluating a time-dependent variable, which was the cross-product of log-transformed 25(OH)D and time ($P = .58$).

We estimated median survival time and survival curves for participants in each category adjusted for covariates by using direct adjusted survival estimation.[27,28] This method uses Cox proportional hazards regression to estimate probabilities of survival at each follow-up time point for each individual and averages them to obtain an overall survival estimate. We assessed statistical interaction by BMI, tobacco use, cancer stage, season of blood collection, and time between blood collection and cancer diagnosis by entering main effect terms and a cross-product term of log-transformed 25(OH)D and stratification variable into the model, and evaluated likelihood ratio tests.

Pancreatic cancer and stromal cells express *VDR*, which translocates to the nucleus and binds vitamin D response elements to regulate gene expression and mediate the activity of vitamin D. Thus, we examined the association of tagging SNPs in *VDR* with survival by including each three-level genotype as a continuous variable (additive model) in multivariable-adjusted Cox proportional hazards regression models. SNPs were considered statistically significant if $P < .002$ (0.05 divided by 30 genotyped variants). We subsequently examined the joint association of 25(OH)D

and *VDR* genotype in Cox regression models. All analyses were performed with SAS 9.3 statistical package. All *P* values were two sided.

RESULTS

Baseline characteristics of patients with pancreatic cancer by category of prediagnostic 25(OH)D and by cohort are listed in Table 1 and Appendix Table A1 (online only), respectively. Median time between blood draw and pancreatic cancer diagnosis was 6.7 years. Mean prediagnostic 25(OH)D was 24.6 ng/mL, with 33% of patients classified as vitamin D insufficient. Among those with known disease stage, 16.0% had localized disease, 29.6% had locally advanced disease, and 54.3% had metastatic disease. Median survival by cancer stage was 17 months for those with localized disease, 10 months for those with locally advanced disease, and 3 months for those with metastatic disease. At the end of follow-up, 464 patients (94%) were deceased.

Higher plasma 25(OH)D levels were associated with greater survival (*P* trend = .01; Table 2 and Fig 1). Compared with patients with insufficient levels of vitamin D, the multivariable-adjusted HRs for death were 0.79 (95% CI, 0.48 to 1.29) for patients with relative insufficiency and 0.66 (95% CI, 0.49 to 0.90) for patients with sufficient 25(OH)D levels. After adjustment for time between blood collection and diagnosis, BMI, and history of diabetes, our results remained largely unchanged (Table 2). In analyses to compare more extreme values of 25(OH)D, a similar inverse relationship was noted between plasma 25(OH)D and patient survival (Table 3). Compared with those in the bottom quintile of 25(OH)D, patients in the top quintile had a multivariable-adjusted HR for death of 0.68 (95% CI, 0.48 to 0.95). We considered the possible influence of subclinical malignancy on plasma 25(OH)D. After excluding 19 patients who were diagnosed with pancreatic cancer within one year of blood collection, our results were not materially altered (Appendix Table A2, online only).

No statistically significant interactions were observed by BMI, tobacco use, cancer stage, season of blood collection, or time between blood collection and diagnosis (Table 4). A stronger association of 25(OH)D and patient survival was identified in patients with blood collected within 5 years of diagnosis. Among patients with blood collected ≤ 5 years before cancer diagnosis, HR for death was 0.58 (95% CI, 0.35 to 0.98), a comparison of those with sufficient levels and those with insufficient levels of 25(OH)D. We evaluated the association of 25(OH)D and patient survival across the cohort study populations (Fig 2), and noted no statistically significant heterogeneity (*P* heterogeneity = .78). In a comparison of patients with sufficient 25(OH)D levels and patients with insufficient levels of plasma vitamin D, multivariable-adjusted HRs for death in sex-specific meta-analyses were 0.61 (95% CI, 0.42 to 0.89) for females (NHS, WHI, and WHS) and 0.79 (95% CI, 0.45 to 1.39) for males (HPFS and PHS).

We next examined genotypic variation at *VDR* in relation to patient survival. Three tagging SNPs in *VDR* were associated with survival to $P < .05$ in an additive model of inheritance, but no SNP met the predefined significance threshold after multiple hypothesis testing correction (Appendix Table A3, online only). The most significant SNP was rs7299460, which had a per-allele HR for death of 0.80 (95% CI, 0.68 to 0.95; $P = .01$). In an exploratory analysis, patients who were homozygous recessive at rs7299460 and with sufficient 25(OH)D had the best overall survival, with an HR for death of 0.45 (95% CI, 0.22 to 0.93; Appendix Table A4, online only).

Table 1. Baseline Characteristics of Patients With Pancreatic Cancer by Prediagnostic Plasma 25(OH)D Level

Characteristic	Plasma 25(OH)D		
	Insufficient	Relative Insufficiency	Sufficient
25(OH)D level			
ng/mL	< 20	20 to < 30	≥ 30
nM	< 50	50 to < 75	≥ 75
No. of patients	165	212	116
Age at blood draw, years	63.9 (7.7)	64.0 (8.8)	61.6 (8.8)
Age at diagnosis, years	71.1 (8.1)	71.7 (8.1)	70.2 (8.7)
Female sex	126 (76.4)	152 (71.7)	67 (57.8)
Race/ethnicity			
White	142 (86.1)	188 (88.7)	106 (91.4)
Black	14 (8.5)	4 (1.9)	0 (0)
Other	5 (3.0)	8 (3.8)	2 (1.7)
Missing	4 (2.4)	12 (5.7)	8 (6.9)
Body mass index, kg/m^2	27.2 (5.0)	26.7 (5.5)	24.9 (3.5)
Physical activity, MET h/wk	11.5 (14.7)	16.8 (20.6)	26.9 (29.4)
Diabetes at blood draw	10 (6.1)	12 (5.7)	7 (6.0)
Tobacco use			
Never	65 (39.4)	96 (45.3)	43 (37.1)
Past	69 (41.8)	90 (42.5)	62 (53.4)
Current	29 (17.6)	25 (11.8)	11 (9.5)
Missing	2 (1.2)	1 (0.5)	0 (0)
Multivitamin use	60 (36.4)	104 (49.1)	53 (45.7)
Alcohol, ≥ 1 drink/d	46 (27.9)	45 (21.2)	32 (27.6)
Diagnosis period			
1984-2000	94 (57.0)	111 (52.4)	65 (56.0)
2001-2008	71 (43.0)	101 (47.6)	51 (44.0)
Cancer stage			
Localized	21 (12.7)	25 (11.8)	19 (16.4)
Locally advanced	48 (29.1)	49 (23.1)	23 (19.8)
Metastatic	68 (41.2)	97 (45.8)	55 (47.4)
Unknown	28 (17.0)	41 (19.3)	19 (16.4)

NOTE. Continuous variables reported as mean (standard deviation) and categorical variables reported as No. (%), unless otherwise noted.
Abbreviations: 25(OH)D, 25-hydroxyvitamin D; MET, metabolic equivalent.

DISCUSSION

In this prospective study of patients with pancreatic cancer from five large US cohorts, patients with sufficient levels of prediagnostic 25(OH)D had a 35% lower hazard for death compared with those who were vitamin D deficient. Association of prediagnostic 25(OH)D with survival remained unchanged after consideration of multiple potential confounding factors and seemed to be strongest when blood was collected within five years before pancreatic cancer diagnosis. Although several polymorphisms at the *VDR* locus were nominally associated with survival, no SNP met the threshold for statistical significance after multiple hypothesis testing correction. In aggregate, these results indicate that prediagnostic plasma 25(OH)D is a prognostic factor in patients with pancreatic cancer.

In laboratory models, vitamin D and its analogs have been shown to induce differentiation, promote apoptosis, and inhibit proliferation of pancreatic cancers.[3,5,29-31] Furthermore, recent studies have indicated direct effects of ligand binding to VDR in pancreatic cancer cells, but also indirect effects by VDR

Table 2. HRs for Death Among Patients With Pancreatic Cancer by Plasma 25(OH)D Level

Model	Plasma 25(OH)D			P_{trend}*
	Insufficient	Relative Insufficiency	Sufficient	
25(OH)D level				
ng/mL	< 20	20 to < 30	≥ 30	
nM	< 50	50 to < 75	≥ 75	
Median survival, months	5	7	8	
Person-months	1,712	2,843	1,348	
Patients/deaths	165/154	212/199	116/111	
HR (95% CI)†	1.0	0.79 (0.48 to 1.29)	0.66 (0.49 to 0.90)	.01
HR (95% CI)‡	1.0	0.83 (0.52 to 1.32)	0.67 (0.49 to 0.92)	.02
HR (95% CI)§	1.0	0.77 (0.46 to 1.29)	0.62 (0.44 to 0.86)	.02

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio.
*Test for trend calculated by entering log-transformed 25(OH)D as a continuous variable in Cox proportional hazards regression models.
†Meta-analysis of cohort-specific HRs (95% CI) from Cox proportional hazards regression models adjusted for age at diagnosis, race and ethnicity (white, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).
‡Model further adjusted for time between blood collection and cancer diagnosis (0 to < 5 years, 5 to < 10 years, or ≥ 10 years).
§Model further adjusted for body mass index (continuous) and history of diabetes (yes or no).

engagement in stromal cells.[6-8] In a study by Sherman and colleagues,[8] VDR acted as a master transcriptional regulator of pancreatic stellate cells, which reside within the tumor microenvironment. Treatment of pancreatic cancer in genetically engineered mice with a vitamin D analog led to remodeling of the stroma, increased concentration of intratumoral chemotherapy, and prolonged survival.

Few studies have investigated whether plasma levels of 25(OH)D predict patient survival. Circulating 25(OH)D is a preferred measure of vitamin D status as a result of its 2-week half-life and its reflection of both vitamin D ingested in the diet and synthesized in the skin.[32] In a retrospective study of 178 patients who were treated at a tertiary cancer center and who underwent vitamin D measurement as part of clinical care, serum 25(OH)D < 20 ng/mL at the initial clinical visit was associated with poor prognosis (HR, 1.99; 95% CI, 1.16 to 3.43) in patients with advanced disease.[12] In contrast, baseline serum 25(OH)D levels were not associated with overall survival in 256 patients with advanced pancreatic cancer who were enrolled in a randomized clinical trial of gemcitabine with or without bevacizumab.[11] However, these studies had relatively small sample sizes, different patient populations, and plasma 25(OH)D measurements made after cancer diagnosis. Because inadequate nutrition and limited outdoor activity as a result of morbidity of recently diagnosed pancreatic cancer can reduce 25(OH)D levels, measured levels likely do not reflect the long-term exposure to vitamin D before acute illness. Although little data are available related to pancreatic cancer, studies of several other malignancies have suggested that single nucleotide variants at *VDR* are associated with patient survival, including in smoking-associated cancers.[33-35]

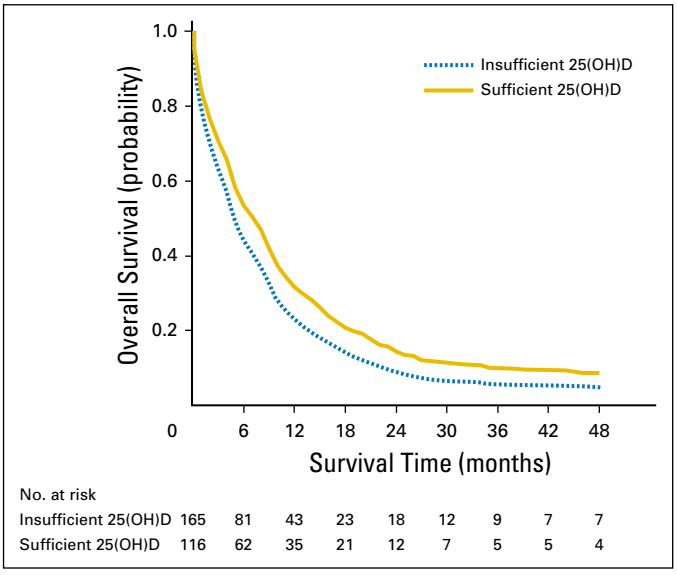


Fig 1. Overall survival curves by prediagnostic plasma 25-hydroxyvitamin D [25(OH)D] among patients with pancreatic cancer.

Median survival times were longer by 3 to 4 months in patients with sufficient circulating 25(OH)D compared with patients with deficient levels. Two multiagent chemotherapy programs have recently been adopted for treatment of patients with metastatic pancreatic cancer, and have improved median overall survival by 1.8 months (gemcitabine plus nab-paclitaxel)[36] and 4.3 months (FOLFIRINOX)[37] compared with single-agent gemcitabine. Whether addition of vitamin D or its analogs to systemic chemotherapy can further improve patient outcomes is currently being investigated (eg, ClinicalTrials.gov NCT02030860).

Several strengths of this study are notable, including large sample size, inclusion of patients with all stages of disease and from all geographic regions of the United States, and a prospective cohort study design. An important aspect of the prospective cohort design is its ability to fully capture the spectrum of patients with pancreatic cancer in terms of disease aggressiveness and stage of disease, as individuals are enrolled before their diagnosis and are not identified at selected tertiary care centers. Of note, survival times and stage distribution for patients in the five cohorts were similar to 121,713 patients who were included in the National Cancer Database, which is thought to capture 76% of patient cases of pancreatic cancer diagnosed in the United States each year.[38] The prospective cohort design reduces bias that results from reverse causation, as blood samples were collected before nutritional deficiencies and limited performance status that commonly develop at the time of pancreatic cancer diagnosis. Furthermore,

Table 3. HRs for Death Among Patients With Pancreatic Cancer by Quintile of Plasma 25(OH)D

Model	Quintile of Plasma 25(OH)D*				
	1	2	3	4	5
Median 25(OH)D, nM	35.7	48.2	58.3	68.8	88.1
Person-months	1,024	1,172	1,371	1,165	1,171
Patients/deaths	97/92	99/91	101/94	99/95	97/92
HR (95% CI)†	1.0	0.91 (0.64 to 1.30)	0.72 (0.51 to 1.01)	0.74 (0.50 to 1.09)	0.68 (0.48 to 0.95)
HR (95% CI)‡	1.0	0.91 (0.64 to 1.31)	0.77 (0.54 to 1.09)	0.76 (0.53 to 1.09)	0.66 (0.47 to 0.94)
HR (95% CI)§	1.0	0.96 (0.67 to 1.39)	0.74 (0.52 to 1.06)	0.76 (0.51 to 1.13)	0.66 (0.46 to 0.95)

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio.
*Cohort-specific quintile ranges of plasma 25(OH)D: Health Professionals Follow-up Study (< 46.9, 46.9 to < 62.0, 62.0 to < 72.3, 72.3 to < 83.9, ≥ 83.9), Nurses' Health Study (< 44.3, 44.3 to < 54.1, 54.1 to < 64.7, 64.7 to < 82.9, ≥ 82.9), Physicians' Health Study (< 45.0, 45.0 to < 58.3, 58.3 to < 68.6, 68.6 to < 86.9, ≥ 86.9), Women's Health Initiative (< 38.2, 38.2 to < 49.4, 49.4 to < 59.7, 59.7 to < 68.9, ≥ 68.9), and Women's Health Study (< 44.9, 44.9 to < 56.4, 56.4 to < 61.8, 61.8 to < 79.7, ≥ 79.7).
†Meta-analysis of cohort-specific HRs (95% CI) from Cox proportional hazards regression models adjusted for age at diagnosis, race and ethnicity (white, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).
‡Model further adjusted for time between blood collection and cancer diagnosis (0 to < 5 years, 5 to < 10 years, or ≥ 10 years).
§Model further adjusted for body mass index (continuous) and history of diabetes (yes or no).

exclusion of patients who were diagnosed with pancreatic cancer within 1 year of blood collection did not materially alter our results. Circulating 25(OH)D was measured in a single laboratory as a single batch, with low coefficients of variance for blinded, replicate quality control samples, and extensive covariate data from all five cohorts allowed for rigorous control of potential confounding factors and evaluation of effect modification.

Limitations of the current study must also be considered. Among patients with pancreatic cancer, treatment programs likely varied, and we could not control for differences in treatment because this information was not collected in our cohorts. Nevertheless, chemotherapy and radiation have had only modest impact on patient survival,[2] and treatment programs were unlikely to have varied meaningfully by baseline 25(OH)D measured years before diagnosis. We used overall mortality data in our analyses, as opposed to pancreatic cancer–specific mortality; however, pancreatic cancer is a highly lethal malignancy, with overall cure rates of < 5%, such that overall mortality is a good surrogate for cancer-specific mortality. Although circulating 25(OH)D was measured at a single time point, we have previously shown a high correlation of 0.70 for repeated measures of plasma 25(OH)D within individuals over time,[39] which suggests that a single measurement is a reasonable proxy for long-term levels of 25(OH)D. We cannot rule out that our findings may be influenced, in part, by residual confounding or that prediagnostic plasma 25(OH)D levels may mark overall health status impacting patient survival. Nonetheless, we included multiple possible confounding covariates in multivariable models without observing meaningful changes in risk estimates.

Table 4. HRs for Death Among Patients With Pancreatic Cancer by Plasma 25(OH)D Level Stratified by Covariates

Stratification Covariate	No. of Patients	Plasma 25(OH)D, HR (95% CI)*			*P* Interaction
		Insufficient	Relative Insufficiency	Sufficient	
BMI, kg/m^2					.44
< 25.0	216	1.0	0.92 (0.63 to 1.35)	0.86 (0.57 to 1.30)	
25.0-29.9	182	1.0	0.67 (0.46 to 0.97)	0.70 (0.44 to 1.12)	
≥ 30.0	95	1.0	0.64 (0.35 to 1.17)	0.37 (0.15 to 0.93)	
Tobacco use					.60
Never	204	1.0	0.87 (0.60 to 1.25)	0.64 (0.41 to 1.01)	
Past	221	1.0	0.68 (0.48 to 0.96)	0.68 (0.46 to 1.00)	
Current	65	1.0	1.12 (0.47 to 2.72)	1.17 (0.41 to 3.32)	
Cancer stage					.13
Localized	65	1.0	0.51 (0.21 to 1.24)	1.07 (0.39 to 2.96)	
Locally advanced	120	1.0	0.91 (0.53 to 1.56)	0.67 (0.35 to 1.27)	
Metastatic	220	1.0	0.91 (0.65 to 1.28)	0.63 (0.41 to 0.96)	
Season of blood draw					.66
Summer/autumn	207	1.0	0.91 (0.65 to 1.26)	0.76 (0.48 to 1.23)	
Winter/spring	286	1.0	0.71 (0.52 to 0.98)	0.64 (0.45 to 0.90)	
Time of blood draw to diagnosis, years					.20
≤ 5	165	1.0	0.64 (0.43 to 0.95)	0.58 (0.35 to 0.98)	
5-10	183	1.0	1.03 (0.70 to 1.50)	0.75 (0.47 to 1.19)	
> 10	145	1.0	0.88 (0.54 to 1.44)	1.02 (0.60 to 1.75)	

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; BMI, body mass index; HR, hazard ratio.
*Adjusted for age at diagnosis, cohort (also adjusts for sex), race and ethnicity (white, black, other, or unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).

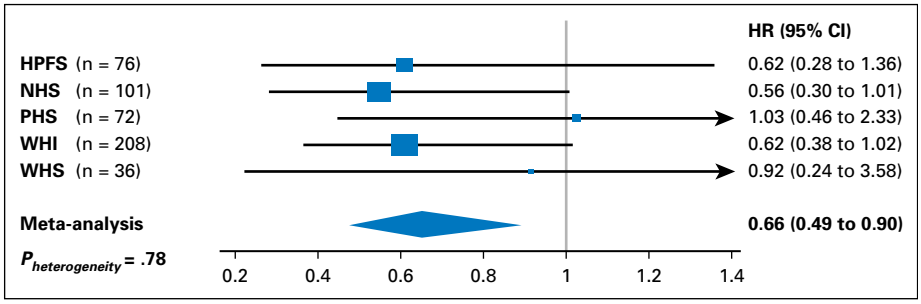


Fig 2. Forest plot and meta-analysis of hazard ratios (HRs) for death among patients with pancreatic cancer, comparing those with sufficient levels of 25-hydroxyvitamin D with those with insufficient levels in the Health Professionals Follow-up Study (HPFS), Nurses' Health Study (NHS), Physicians' Health Study (PHS), Women's Health Initiative (WHI), and Women's Health Study (WHS). Solid squares and horizontal lines correspond to the cohort-specific multivariable-adjusted hazard ratios and 95% CIs. Area of the solid square reflects the cohort-specific weight (inverse of the variance). Diamond represents the meta-analysis multivariable-adjusted HR and 95% CI. Vertical line indicates an odds ratio of 1.0. HRs adjusted for age at diagnosis, race and ethnicity (white, black, other, unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).

Finally, our study participants were predominantly individuals of European descent. African Americans have higher rates of vitamin D deficiency[40] and pancreatic cancer mortality.[41,42] Additional studies of vitamin D levels and pancreatic cancer survival are warranted in racially diverse patient populations.[43]

Higher prediagnostic plasma levels of 25(OH)D were associated with a statistically significant improvement in survival among patients with pancreatic cancer who were enrolled in five large, prospective cohorts. When considering these findings together with previously reported preclinical data in pancreatic cancer models, agonists of the vitamin D receptor are a potentially attractive therapeutic approach for investigation in this highly lethal malignancy.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

AUTHOR CONTRIBUTIONS

Conception and design: Chen Yuan, Barbara B. Cochrane, JoAnn E. Manson, Brian M. Wolpin
Financial support: Chen Yuan, Kimmie Ng, Shuji Ogino, Charles S. Fuchs, Brian M. Wolpin
Administrative support: Chen Yuan, JoAnn E. Manson, Charles S. Fuchs
Provision of study materials or patients: Chen Yuan, Rowan T. Chlebowski, Charles S. Fuchs
Collection and assembly of data: Chen Yuan, Howard D. Sesso, Barbara B. Cochrane, Rowan T. Chlebowski, JoAnn E. Manson, Charles S. Fuchs, Brian M. Wolpin
Data analysis and interpretation: Chen Yuan, Zhi Rong Qian, Ana Babic, Vicente Morales-Oyarvide, Douglas A. Rubinson, Peter Kraft, Kimmie Ng, Ying Bao, Edward L. Giovannucci, Shuji Ogino, Meir J. Stampfer, John Michael Gaziano, Howard D. Sesso, Julie E. Buring, Rowan T. Chlebowski, Linda G. Snetselaar, JoAnn E. Manson, Charles S. Fuchs, Brian M. Wolpin
Manuscript writing: All authors
Final approval of manuscript: All authors

REFERENCES

1. Siegel RL, Miller KD, Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66:7-30, 2016
2. Hidalgo M: Pancreatic cancer. N Engl J Med 362:1605-1617, 2010
3. Colston KW, James SY, Ofori-Kuragu EA, et al: Vitamin D receptors and anti-proliferative effects of vitamin D derivatives in human pancreatic carcinoma cells in vivo and in vitro. Br J Cancer 76:1017-1020, 1997
4. Persons KS, Eddy VJ, Chadid S, et al: Anti-growth effect of 1,25-dihydroxyvitamin D_3-3-bromoacetate alone or in combination with 5-amino-imidazole-4-carboxamide-1-beta-4-ribofuranoside in pancreatic cancer cells. Anticancer Res 30: 1875-1880, 2010
5. Schwartz GG, Eads D, Rao A, et al: Pancreatic cancer cells express 25-hydroxyvitamin D-1 alpha-hydroxylase and their proliferation is inhibited by the prohormone 25-hydroxyvitamin D_3. Carcinogenesis 25:1015-1026, 2004
6. Arensman MD, Nguyen P, Kershaw KM, et al: Calcipotriol targets LRP6 to inhibit Wnt signaling in pancreatic cancer. Mol Cancer Res 13:1509-1519, 2015
7. Li Z, Jia Z, Gao Y, et al: Activation of vitamin D receptor signaling downregulates the expression of nuclear FOXM1 protein and suppresses pancreatic cancer cell stemness. Clin Cancer Res 21:844-853, 2015
8. Sherman MH, Yu RT, Engle DD, et al: Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. Cell 159:80-93, 2014
9. Wolpin BM, Ng K, Bao Y, et al: Plasma 25-hydroxyvitamin D and risk of pancreatic cancer. Cancer Epidemiol Biomarkers Prev 21:82-91, 2012
10. Stolzenberg-Solomon RZ, Jacobs EJ, Arslan AA, et al: Circulating 25-hydroxyvitamin D and risk of pancreatic cancer: Cohort Consortium Vitamin D Pooling Project of Rarer Cancers. Am J Epidemiol 172:81-93, 2010
11. Van Loon K, Owzar K, Jiang C, et al: 25-Hydroxyvitamin D levels and survival in advanced pancreatic cancer: Findings from CALGB 80303 (Alliance). J Natl Cancer Inst 106:dju185, 2014
12. Cho M, Peddi PF, Ding K, et al: Vitamin D deficiency and prognostics among patients with pancreatic adenocarcinoma. J Transl Med 11:206, 2013
13. Giovannucci E, Ascherio A, Rimm EB, et al: Physical activity, obesity, and risk for colon cancer and adenoma in men. Ann Intern Med 122:327-334, 1995
14. Colditz GA, Hankinson SE: The Nurses' Health Study: Lifestyle and health among women. Nat Rev Cancer 5:388-396, 2005
15. Manson JE, Grobbee DE, Stampfer MJ, et al: Aspirin in the primary prevention of angina pectoris in a randomized trial of United States physicians. Am J Med 89:772-776, 1990
16. Langer RD, White E, Lewis CE, et al: The Women's Health Initiative Observational Study: Baseline characteristics of participants and reliability of baseline measures. Ann Epidemiol 13:S107-S121, 2003 (suppl 9)
17. Cook NR, Lee IM, Gaziano JM, et al: Low-dose aspirin in the primary prevention of cancer: The Women's Health Study: A randomized controlled trial. JAMA 294:47-55, 2005
18. Rich-Edwards JW, Corsano KA, Stampfer MJ: Test of the National Death Index and Equifax Nationwide Death Search. Am J Epidemiol 140: 1016-1019, 1994
19. Mayers JR, Wu C, Clish CB, et al: Elevation of circulating branched-chain amino acids is an early

event in human pancreatic adenocarcinoma development. Nat Med 20:1193-1198, 2014

20. Ahn J, Albanes D, Berndt SI, et al: Vitamin D-related genes, serum vitamin D concentrations and prostate cancer risk. Carcinogenesis 30:769-776, 2009

21. Bischoff-Ferrari HA, Giovannucci E, Willett WC, et al: Estimation of optimal serum concentrations of 25-hydroxyvitamin D for multiple health outcomes. Am J Clin Nutr 84:18-28, 2006 [Errata: Am J Clin Nutr 84:1253, 2006; and Am J Clin Nutr 86:809, 2007]

22. Holick MF: Vitamin D deficiency. N Engl J Med 357:266-281, 2007

23. DerSimonian R, Laird N: Meta-analysis in clinical trials. Control Clin Trials 7:177-188, 1986

24. Cochran WG: The combination of estimates from different experiments. Biometrics 10:101-129, 1954

25. Yuan C, Bao Y, Wu C, et al: Prediagnostic body mass index and pancreatic cancer survival. J Clin Oncol 31:4229-4234, 2013

26. Yuan C, Rubinson DA, Qian ZR, et al: Survival among patients with pancreatic cancer and long-standing or recent-onset diabetes mellitus. J Clin Oncol 33:29-35, 2015

27. Ghali WA, Quan H, Brant R, et al: Comparison of 2 methods for calculating adjusted survival curves from proportional hazards models. JAMA 286: 1494-1497, 2001

28. Makuch RW: Adjusted survival curve estimation using covariates. J Chronic Dis 35:437-443, 1982

29. Kawa S, Yoshizawa K, Tokoo M, et al: Inhibitory effect of 220-oxa-1,25-dihydroxyvitamin D3 on the proliferation of pancreatic cancer cell lines. Gastroenterology 110:1605-1613, 1996

30. Pettersson F, Colston KW, Dalgleish AG: Differential and antagonistic effects of 9-cis-retinoic acid and vitamin D analogues on pancreatic cancer cells in vitro. Br J Cancer 83:239-245, 2000

31. Zugmaier G, Jäger R, Grage B, et al: Growth-inhibitory effects of vitamin D analogues and retinoids on human pancreatic cancer cells. Br J Cancer 73:1341-1346, 1996

32. Zerwekh JE: Blood biomarkers of vitamin D status. Am J Clin Nutr 87:1087S-1091S, 2008

33. Deschasaux M, Souberbielle JC, Latino-Martel P, et al: Prospective associations between vitamin D status, vitamin D-related gene polymorphisms, and risk of tobacco-related cancers. Am J Clin Nutr 102: 1207-1215, 2015

34. Heist RS, Zhou W, Wang Z, et al: Circulating 25-hydroxyvitamin D, VDR polymorphisms, and survival in advanced non-small-cell lung cancer. J Clin Oncol 26:5596-5602, 2008

35. Shui IM, Mucci LA, Kraft P, et al: Vitamin D-related genetic variation, plasma vitamin D, and risk of lethal prostate cancer: A prospective nested case-control study. J Natl Cancer Inst 104:690-699, 2012

36. Von Hoff DD, Ervin T, Arena FP, et al: Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. N Engl J Med 369: 1691-1703, 2013

37. Conroy T, Desseigne F, Ychou M, et al: FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 364:1817-1825, 2011

38. Bilimoria KY, Bentrem DJ, Ko CY, et al: Validation of the 6th edition AJCC Pancreatic Cancer Staging System: Report from the National Cancer Database. Cancer. 110:738-744, 2007

39. Platz EA, Leitzmann MF, Hollis BW, et al: Plasma 1,25-dihydroxy- and 25-hydroxyvitamin D and subsequent risk of prostate cancer. Cancer Causes Control 15:255-265, 2004

40. Ginde AA, Liu MC, Camargo CA Jr. Demographic differences and trends of vitamin D insufficiency in the US population, 1988-2004. Arch Intern Med 169:626-632, 2009

41. National Cancer Institute: SEER Cancer Statistics Review (CSR), 1975-2013. http://seer.cancer.gov/csr/1975_2013/

42. Khawja SN, Mohammed S, Silberfein EJ, et al: Pancreatic cancer disparities in African Americans. Pancreas 44:522-527, 2015

43. Grant WB, Peiris AN: Differences in vitamin D status may account for unexplained disparities in cancer survival rates between African and white Americans. Dermatoendocrinol 4:85-94, 2012

Affiliations

Chen Yuan, Zhi Rong Qian, Ana Babic, Vicente Morales-Oyarvide, Douglas A. Rubinson, Kimmie Ng, Shuji Ogino, Charles S. Fuchs, and Brian M. Wolpin, Dana-Farber Cancer Institute and Harvard Medical School; Peter Kraft, Edward L. Giovannucci, Shuji Ogino, Meir J. Stampfer, Howard D. Sesso, Julie E. Buring, and JoAnn E. Manson, Harvard School of Public Health; Ying Bao, Edward L. Giovannucci, Shuji Ogino, Meir J. Stampfer, John Michael Gaziano, Howard D. Sesso, JoAnn E. Manson, and Charles S. Fuchs, Brigham and Women's Hospital and Harvard Medical School; John Michael Gaziano, Massachusetts Veterans Epidemiology Research and Information Center, VA Boston Healthcare System, Boston, MA; Barbara B. Cochrane, University of Washington School of Nursing, Seattle, WA; Rowan T. Chlebowski, Los Angeles Biomedical Research Institute at Harbor-University of California, Los Angeles Medical Center, Torrance, CA; and Linda G. Snetselaar, University of Iowa College of Public Health, Iowa City, IA.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Prediagnostic Plasma 25-Hydroxyvitamin D and Pancreatic Cancer Survival

The following represents disclosure information provided by authors of this manuscript. All relationships are considered compensated. Relationships are self-held unless noted. I = Immediate Family Member, Inst = My Institution. Relationships may not relate to the subject matter of this manuscript. For more information about ASCO's conflict of interest policy, please refer to www.asco.org/rwc or jco.ascopubs.org/site/ifc.

Chen Yuan
No relationship to disclose

Zhi Rong Qian
No relationship to disclose

Ana Babic
Stock or Other Ownership: Biogen Idec

Vicente Morales-Oyarvide
No relationship to disclose

Douglas A. Rubinson
No relationship to disclose

Peter Kraft
Consulting or Advisory Role: Merck

Kimmie Ng
Honoraria: Sage Publications, Prime Oncology
Consulting or Advisory Role: Genentech, Havas Life Metro, CBPartners, Defined Health
Research Funding: Genentech (Inst), Pharmavite (Inst)

Ying Bao
No relationship to disclose

Edward L. Giovannucci
No relationship to disclose

Shuji Ogino
No relationship to disclose

Meir J. Stampfer
No relationship to disclose

John Michael Gaziano
No relationship to disclose

Howard D. Sesso
Research Funding: Pfizer (Inst)

Julie E. Buring
No relationship to disclose

Barbara B. Cochrane
No relationship to disclose

Rowan T. Chlebowski
Consulting or Advisory Role: Pfizer, AstraZeneca, Novartis, Amgen, Genomic Health International, Novo Nordisk
Speakers' Bureau: Novartis, Genentech
Travel, Accommodations, Expenses: Pfizer

Linda G. Snetselaar
No relationship to disclose

JoAnn E. Manson
No relationship to disclose

Charles S. Fuchs
Consulting or Advisory Role: Sanofi, Amgen, Eli Lilly, Pfizer, Genentech, Celgene, Merck, Gilead Sciences, Macrogenics, Dicerna, Five Prime Therapeutics, Bristol-Myers Squibb

Brian M. Wolpin
Consulting or Advisory Role: Genentech

Acknowledgment

We thank the participants and staff of the Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative, and Women's Health Study for their valuable contributions, as well as the following state cancer registries for their help: Alabama, Arizona, Arkansas, California, Colorado, Connecticut, Delaware, Florida, Georgia, Idaho, Illinois, Indiana, Iowa, Kentucky, Louisiana, Maine, Massachusetts, Michigan, Nebraska, New Hampshire, New Jersey, New York, North Carolina, North Dakota, Ohio, Oklahoma, Oregon, Pennsylvania, Rhode Island, South Carolina, Tennessee, Texas, Virginia, Washington, and Wyoming.

Appendix

Table A1. Baseline Characteristics of Patients With Pancreatic Cancer by Cohort

Characteristic	HPFS	NHS	PHS	WHI	WHS	Total
No. of patients	76	101	72	208	36	493
Plasma 25(OH)D, nM	68.5 (24.3)	65.4 (25.7)	65.8 (22.1)	55.2 (19.6)	60.4 (19.5)	61.3 (22.7)
Age at blood draw, years	65.6 (7.8)	60.5 (6.7)	56.9 (8.6)	67.0 (7.3)	58.9 (8.9)	63.4 (8.5)
Age at diagnosis, years	72.7 (8.4)	70.7 (7.0)	72.0 (9.4)	71.9 (7.5)	63.1 (9.1)	71.2 (8.3)
Female sex	0 (0)	101 (100)	0 (0)	208 (100)	36 (100)	345 (70.0)
Race/ethnicity						
White	71 (93.4)	100 (99.0)	53 (73.6)	178 (85.6)	34 (94.4)	436 (88.4)
Black	1 (1.3)	1 (1.0)	1 (1.4)	14 (6.7)	1 (2.8)	18 (3.7)
Other	1 (1.3)	0 (0)	0 (0)	14 (6.7)	0 (0)	15 (3.0)
Missing	3 (3.9)	0 (0)	18 (25.0)	2 (1.0)	1 (2.8)	24 (4.9)
Body mass index, kg/m^2	25.7 (3.3)	25.6 (5.0)	25.7 (2.9)	27.4 (5.9)	26.4 (5.3)	26.4 (5.0)
Physical activity, MET h/wk	36.9 (38.5)	15.4 (17.8)	12.0 (11.2)	13.0 (13.2)	17.8 (21.8)	17.4 (22.1)
History of diabetes	3 (3.9)	5 (5.0)	3 (4.2)	15 (7.2)	3 (8.3)	29 (5.9)
Tobacco use						
Never	26 (34.2)	40 (39.6)	29 (40.3)	96 (46.2)	13 (36.1)	204 (41.4)
Past	42 (55.3)	41 (40.6)	30 (41.7)	92 (44.2)	16 (44.4)	221 (44.8)
Current	8 (10.5)	20 (19.8)	13 (18.1)	17 (8.2)	7 (19.4)	65 (13.2)
Missing	0 (0)	0 (0)	0 (0)	3 (1.4)	0 (0)	3 (0.6)
Multivitamin use	41 (53.9)	46 (45.5)	21 (29.2)	99 (47.6)	10 (27.8)	217 (44.0)
Alcohol (≥ 1 drink/d)	32 (42.1)	24 (23.8)	24 (33.3)	36 (17.3)	7 (19.4)	123 (24.9)
Median time blood draw to diagnosis, years	6.5	10.6	16.6	5.2	4.1	6.7
Diagnosis period						
1984-2000	39 (51.3)	52 (51.5)	45 (62.5)	103 (49.5)	31 (86.1)	270 (54.8)
2001-2008	37 (48.7)	49 (48.5)	27 (37.5)	105 (50.5)	5 (13.9)	223 (45.2)
Cancer stage						
Localized	11 (14.5)	19 (18.8)	14 (19.4)	13 (6.3)	8 (22.2)	65 (13.2)
Locally advanced	11 (14.5)	12 (11.9)	14 (19.4)	77 (37.0)	6 (16.7)	120 (24.3)
Metastatic	33 (43.4)	44 (43.6)	32 (44.4)	91 (43.8)	20 (55.6)	220 (44.6)
Unknown	21 (27.6)	26 (25.7)	12 (16.7)	27 (13.0)	2 (5.6)	88 (17.8)
Median survival time, months						
All patients	5	5	6.5	8	5	6
By stage						
Localized	14	21	14.5	25.0	11.5	17
Locally advanced	10	9	8.5	12.0	10.5	10
Metastatic	4	3	3.5	4.0	4.5	3
Unknown	4	6	7.0	5.0	3.0	5

NOTE. Continuous variables reported as mean (standard deviation) and categorical variables reported as No. (%), unless otherwise noted.
Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HPFS, Health Professionals Follow-up Study; MET, metabolic equivalent; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study.

Table A2. HRs for Death Among Patients With Pancreatic Cancer by Plasma 25(OH)D Level, Excluding Patients Diagnosed Within 1 Year of Blood Draw

Model	Plasma 25(OH)D			P_{trend}*
	Insufficient	Relative Insufficiency	Sufficient	
Range				
ng/mL	< 20	20 to < 30	≥ 30	
nM	< 50	50 to < 75	≥ 75	
Median survival, months	5	7	8	
Person-months	1,662	2,541	1,325	
Patients/deaths	159/148	201/189	113/108	
HR (95% CI)†	1.0	0.84 (0.53 to 1.33)	0.62 (0.45 to 0.85)	.02
HR (95% CI)‡	1.0	0.87 (0.55 to 1.36)	0.63 (0.45 to 0.87)	.03
HR (95% CI)§	1.0	0.75 (0.41 to 1.36)	0.59 (0.41 to 0.84)	.04

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; HR, hazard ratio.
*Test for trend calculated by entering log-transformed 25(OH)D as a continuous variable in Cox proportional hazards regression models.
†Meta-analysis of cohort-specific HRs (95% CI) from Cox proportional hazards regression models adjusted for age at diagnosis, race and ethnicity (white, black, other, or unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).
‡Model further adjusted for time between blood collection and cancer diagnosis (0 to < 5 years, 5 to < 10 years, or ≥ 10 years).
§Model further adjusted for body mass index (continuous) and history of diabetes (yes or no).

Table A3. SNPs in *VDR* Ranked by Association *P* Value With Overall Survival Among Patients With Pancreatic Cancer

SNP*	No. of Patients	Minor Allele Frequency, %	Additive Model	
			HR (95% CI)	*P*
rs7299460	391	0.30	0.80 (0.68 to 0.95)	.01
rs11568820	388	0.22	0.79 (0.65 to 0.96)	.02
rs4334089	388	0.23	0.81 (0.68 to 0.98)	.03
rs886441	392	0.18	0.82 (0.67 to 0.99)	.04
rs2853564	396	0.38	1.16 (0.99 to 1.35)	.07
rs2239179	392	0.44	1.14 (0.99 to 1.33)	.08
rs2239182	392	0.47	0.89 (0.77 to 1.03)	.12
rs7295021	395	0.20	0.86 (0.70 to 1.05)	.13
rs11168293	392	0.34	1.13 (0.96 to 1.32)	.15
rs2239186	395	0.20	0.89 (0.74 to 1.06)	.20
rs7132324	391	0.35	1.10 (0.94 to 1.29)	.24
rs7963776	388	0.46	0.91 (0.79 to 1.06)	.24
rs2254210	396	0.39	1.09 (0.94 to 1.26)	.28
rs2189480	390	0.34	0.92 (0.79 to 1.08)	.30
rs2283342	392	0.15	0.90 (0.72 to 1.11)	.31
rs4760648	391	0.43	0.92 (0.78 to 1.08)	.32
rs10875693	389	0.33	1.08 (0.92 to 1.27)	.35
rs2238136	393	0.27	0.92 (0.78 to 1.09)	.35
rs11574077	391	0.05	1.18 (0.83 to 1.66)	.36
rs1859281	395	0.07	1.13 (0.83 to 1.53)	.44
rs1544410	359	0.37	1.05 (0.90 to 1.23)	.53
rs7310552	393	0.40	1.04 (0.88 to 1.22)	.64
rs10747524	329	0.49	0.97 (0.81 to 1.16)	.71
rs2107301	394	0.28	0.97 (0.83 to 1.14)	.71
rs11168275	392	0.24	0.97 (0.81 to 1.16)	.73
rs2544037	394	0.42	1.02 (0.87 to 1.18)	.84
rs11574032	297	0.09	1.02 (0.75 to 1.40)	.89
rs12721364	391	0.14	1.01 (0.81 to 1.26)	.93
rs11574143	391	0.11	1.01 (0.80 to 1.27)	.96
rs3819545	393	0.39	1.00 (0.86 to 1.17)	.96

Abbreviations: HR, hazard ratio; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.
*Effect of each minor allele of the SNP on survival in Cox proportional hazards regression models adjusted for age at diagnosis (years, continuous), cohort (also adjusts for sex), race and ethnicity (white, black, other, or unknown), smoking status (never, past, current, or missing), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).

Table A4. HRs (95% CI) for Death Among Patients With Pancreatic Cancer by Plasma 25(OH)D and Stratified by rs7299460 Genotypes in *VDR*

			Plasma 25(OH)D, HR (95% CI)		
SNP	Genotype	No.	Insufficient	Relative Insufficiency	Sufficient
rs7299460	GG	194	1.0	0.97 (0.69 to 1.38)	0.78 (0.51 to 1.18)
	AG	162	0.84 (0.57 to 1.25)	0.67 (0.46 to 0.98)	0.85 (0.56 to 1.30)
	AA	35	0.71 (0.31 to 1.59)	0.62 (0.33 to 1.16)	0.45 (0.22 to 0.93)

NOTE. Adjusted for age at diagnosis (years, continuous), cohort (also adjusts for sex), race and ethnicity (white, black, other, or unknown), smoking status (never, past, current, or missing), month of blood draw (2-month intervals), stage at diagnosis (localized, locally advanced, metastatic, or unknown), and year of diagnosis (1984-2000 or 2001-2008).

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; SNP, single nucleotide polymorphism; VDR, vitamin D receptor.

Title:

Pancreatic cancer risk associated with prediagnostic plasma levels of leptin and leptin receptor genetic polymorphisms

Authors:

Ana Babic[1*], Ying Bao[2*], Zhi Rong Qian[1], Chen Yuan[1], Edward L. Giovannucci[2,3,4], Hugues Aschard[3], Peter Kraft[3,5], Laufey Amundadottir[6], Rachael Stolzenberg-Solomon[7], Vicente Morales-Oyarvide[1], Kimmie Ng[1], Meir J. Stampfer[2,3,4], Shuji Ogino[1,8], Julie E. Buring[9,10], Howard D. Sesso[3,9], John Michael Gaziano[9,11], Nader Rifai[12], Michael N. Pollak[13], Matthew L. Anderson[14], Barbara B. Cochrane[15], Juhua Luo[16], JoAnn E. Manson[2,3,13], Charles S. Fuchs[1,2], Brian M. Wolpin[1]

Author affiliations:

[1] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA
[2] Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA
[3] Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, MA
[4] Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA
[5] Department of Biostatistics, Harvard T.H. Chan School of Public Health, Boston, MA
[6] Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD
[7] Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, DHHS, Bethesda, MD.
[8] Division of MPE Molecular Pathological Epidemiology, Department of Pathology, Brigham and Women's Hospital, Boston, MA
[9] Division of Preventive Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA
[10] Department of Ambulatory Care and Prevention, Harvard Medical School, Boston, MA
[11] Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC), VA Boston Healthcare System
[12] Department of Laboratory Medicine, Children's Hospital Boston, Boston, MA
[13] Cancer Prevention Research Unit, Department of Oncology, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

[14] Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, TX
[15] University of Washington School of Nursing, Seattle, WA
[16] Department of Community Medicine, West Virginia University, Morgantown, WV
* Co-first authors, contributed equally.

Running title: Prediagnostic Plasma Leptin and Pancreatic Cancer Risk

Keywords: pancreatic cancer, obesity, leptin

Grant Support

HPFS is supported by NIH grant UM1 CA167552. NHS is supported by NIH grants UM1 CA186107, P01 CA87969, and R01 CA49449. PHS is supported by NIH grants CA 97193, CA 34944, CA 40360, HL 26490, and HL 34595. The WHI program is funded by the National Heart, Lung, and Blood Institute, National Institutes of Health, U.S. Department of Health and Human Services through contracts HHSN268201600018C, HHSN268201600001C, HHSN268201600002C, HHSN268201600003C, and HHSN268201600004C. The WHS is supported by grants CA047988, HL043851, HL080467, HL099355, and UM1 CA182913 from the National Institutes of Health (Bethesda, MD).

Additional support from NIH KL2 TR001100, NIH U54 CA155626 and NIH to P30 DK046200 to Y. Bao; NIH K07 CA148894 to K. Ng; from NCI R35 CA197735 to S. Ogino; from the Robert T. and Judith B. Hale Fund for Pancreatic Cancer, Perry S. Levy Fund for Gastrointestinal Cancer Research, Pappas Family Research Fund for Pancreatic Cancer, NIH R01 CA124908, and NIH P50 CA127003 to C.S. Fuchs; and from NIH/NCI U01 CA210171, Department of Defense CA130288, Lustgarten Foundation, Pancreatic Cancer Action Network,

Noble Effort Fund, Peter R. Leavitt Family Fund, Wexler Family Fund, and Promises for Purple to B.M. Wolpin.

The study sponsors had no role in the design of the study; the collection, analysis, and interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

Corresponding Author: Brian M. Wolpin, MD, MPH, 450 Brookline Avenue, Boston, MA 02215; phone: (617) 632–6942; fax: (617) 632-5370; e-mail: bwolpin@partners.org

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed

Word count: 3,065

Number of tables: 3

Number of figures: 2

Number of references: 50

ABSTRACT

Leptin is an adipokine involved in regulating energy balance which has been identified as a potential biological link in development of obesity-associated cancers, such as pancreatic cancer. In this prospective, nested case-control study of 470 cases and 1094 controls from five U.S. cohorts, we used conditional logistic regression to evaluate pancreatic cancer risk by prediagnostic plasma leptin, adjusting for race/ethnicity, diabetes, body-mass index, physical activity, plasma C-peptide, adiponectin and 25-hydroxyvitamin D. Due to known differences in leptin levels by gender, analyses were conducted separately for men and women. We also evaluated associations between 32 tagging single nucleotide polymorphisms (SNPs) in the leptin receptor (*LEPR*) gene and pancreatic cancer risk. Leptin levels were higher in female versus male control participants (median, 20.8 vs. 6.7μg/mL; $P<0.0001$). Among men, plasma leptin was positively associated with pancreatic cancer risk, and those in the top quintile had a multivariable-adjusted odds ratio (OR) of 3.02 (95% CI, 1.27-7.16; $P_{trend}=0.02$) compared to men in the bottom quintile. Among women, circulating leptin was not associated with pancreatic cancer risk ($P_{trend}=0.21$). Results were similar across cohorts ($P_{heterogeneity}=0.88$ for two male cohorts and 0.35 for three female cohorts). In genetic analyses, rs10493380 in *LEPR* was associated with increased pancreatic cancer risk among women, with an OR per minor allele of 1.54 (95% CI, 1.18-2.02; multiple hypothesis-corrected $P=0.03$). No SNPs were significantly associated with risk in men. In conclusion, higher prediagnostic levels of plasma leptin were associated with an elevated risk of pancreatic cancer among men, but not among women.

INTRODUCTION

Pancreatic cancer is the third-leading cause of cancer-related death in the United States (1). Obesity is associated with increased pancreatic cancer risk (2,3), but the underlying mechanisms are poorly understood. Leptin was initially discovered in studies of obese mice, which were noted to have increased caloric intake. Subsequent studies demonstrated that the underlying cause of the obese phenotype in these mice was a truncating mutation in the gene encoding for leptin (4). Leptin is a hormone synthesized almost exclusively in adipocytes, and plasma levels of leptin in humans are proportional to total body adipose tissue (5). When circulating levels of leptin are increased, downstream signaling is activated through the ObR (HUGO Gene Nomenclature Committee [HGNC] Symbol LEPR) transmembrane receptor on target cells (6). The leptin receptor is present on cells within the hypothalamus, which is the mechanism by which leptin is thought to regulate caloric intake (7). Additionally, leptin receptor expression is distributed widely throughout the body, including on cells within the pancreas (8). Leptin receptors are also present on tumor cells and activation of these receptors increases cancer cell proliferation and reduces rates of cancer cell apoptosis (9).

Given its essential role in regulating energy balance, leptin may be an important biological link in the development of obesity-associated malignancies, including pancreatic cancer. To further investigate the role of leptin in pancreatic carcinogenesis, we examined the association between prediagnostic plasma leptin and subsequent pancreatic cancer risk in five prospective cohorts with up to 26 years of follow-up since blood collection. We additionally evaluated the association between polymorphisms in *LEPR* with risk of pancreatic cancer.

METHODS

Study Participants

We pooled blood samples and data from five U.S. prospective cohorts. The Health Professionals Follow-up Study (HPFS) enrolled 51,529 male health professionals aged 40-75 in 1986. The Nurses' Health Study (NHS) enrolled 121,700 female nurses aged 30-55 in 1976. The Physicians' Health Study I (PHS) was a randomized clinical trial of aspirin and β-carotene that enrolled 22,071 healthy male physicians aged 40-84 in 1982. The aspirin component of the trial ended in 1988, while the β-carotene component ended in 1995, and PHS I participants continue follow-up as an observational cohort. The Women's Health Initiative (WHI)-Observational Study enrolled 93,676 postmenopausal women aged 50-79 between 1994 and 1998. The Women's Health Study (WHS) was a randomized clinical trial of low-dose aspirin and vitamin E that enrolled 39,876 healthy female health professionals aged ≥45 in 1992. The trial was completed in 2004 and WHS participants continue to be followed as an observational cohort.

Individual characteristics and lifestyle factors were obtained from baseline questionnaires at enrollment in PHS, WHI, and WHS and from the questionnaires preceding blood draw in HPFS and NHS. Details of these cohorts have been described previously (10-14). The current study was approved by the Human Research Committee at the Brigham and Women's Hospital, Boston, MA, and participants provided informed consent.

Blood Collection and Plasma Assays

Blood samples were collected from 18,225 men in HPFS from 1993-1995, 32,826 women in NHS from 1989-1990, 14,916 men in PHS from 1982-1984, 93,676 women in WHI

from 1994-1998, and 28,345 women in WHS from 1992-1995. Details on blood draw, transportation, and storage have been described previously (12,14-16).

Plasma leptin was assayed in the laboratory of Dr. Nader Rifai (Children's Hospital, Boston, MA), using reagents from R&D Systems (Minneapolis, MN). Measurement of plasma adiponectin and C-peptide were described previously (17) . All samples for leptin and adiponectin were handled identically in a single batch and C-peptide was handled in two batches. Laboratory personnel were blinded to case or control status. The mean intra-assay coefficients of variance for quality control samples were ≤10% for each biomarker.

Pancreatic cancer cases and matched controls

We included cases of pancreatic adenocarcinoma diagnosed through 2008 with prediagnostic blood and no prior history of cancer, except non-melanoma skin cancer. Incident cases were identified by self-report or during follow-up of a participant's death. Deaths were ascertained from next-of-kin or the U.S. postal service and by searching the National Death Index. Medical records of the cases were requested and reviewed by study physicians blinded to exposure data.

Eligible controls were cohort participants who provided blood and were alive and free of cancer at the date of the case's diagnosis. We randomly selected 2-3 controls for each case, matching on year of birth (± 5 years), prospective cohort (which concurrently matched on sex), smoking status (never, past, current), fasting status (fasting, non-fasting), and month/year of blood draw.

For the present analysis, 488 pancreatic cancer cases and 1132 matched controls with plasma were available. Due to concern regarding the possible influence of subclinical

malignancy, we excluded pancreatic cancer cases diagnosed within 1 year of blood draw (n=19) and their matched controls (n=38), resulting in a total of 470 cases and 1094 controls (Supplementary Table 1). Of these 470 cases, 465 (99%) were confirmed by review of medical records, tumor registry data, or death certificates.

Single Nucleotide Polymorphisms (SNP) selection and Genotyping

A total of 39 SNPs in the *LEPR* gene +/- 20kb were selected with the tagger algorithm in Haploview, using r^2=0.8 and minor allele frequency (MAF) ≥5% among Whites from the HapMap Project database. Five SNPs associated with: serum amyloid A (rs1275319, (18)), soluble Ob-R (rs2767485, (19)), and CRP: (rs4420065 (20), rs6700896 (21) and rs1892534 (22) were forced in. From 412 cases (Supplementary Table 1), DNA was extracted from buffy coat using QIAGEN QIAmp and whole genome amplified using GE Healthcare GenomiPhi. Genotyping was performed at Partners HealthCare Center for Personalized Genetic Medicine using a custom-designed Illumina Golden Gate genotyping assay. Seven tagging SNPs were not supported by the platform. One SNP (rs913199) deviated from Hardy-Weinberg Equilibrium at P=0.008. Replicate samples tested for quality control (N=44 groups) had a mean genotype concordance of 98.2%.

Statistical Analysis

Median leptin levels among cases and controls were compared using the Wilcoxon rank-sum test. Since men and women have different distributions of leptin levels (23), we performed separate analyses by gender using pooled gender-specific quintiles from controls. To compute odds ratios (ORs) and 95% confidence intervals (CIs), we used conditional logistic regression. In multivariate models, we adjusted for potential confounding factors, including race (White, Black,

other), multivitamin use (yes, no), diabetes (yes, no), body mass index (BMI, kg/m^2), physical activity (MET-hr/wk), plasma C-peptide (continuous), plasma adiponectin (quartiles, as previous analysis demonstrated non-linear association of adiponectin and pancreatic cancer risk (17)), and plasma 25(OH)D (continuous). *P*-trends were calculated by the Wald test of a score variable that contained median values of quintiles. We also conducted a meta-analysis of cohort-level data among men and women. We calculated ORs for each cohort and then pooled the ORs to compute a summary OR by gender using the random effects model (24). Heterogeneity across studies was tested using the Q statistic (24). To evaluate whether the association between leptin and pancreatic cancer risk was log-linear, we compared the model fit including linear and cubic spline terms to the model fit with only the linear term using the likelihood ratio test (25). We conducted subgroup analyses using unconditional logistic regression adjusted for the matching factors and covariates. Tests for interaction were performed by the Wald test of cross-product terms. We conducted sensitivity analyses excluding diabetics or cases diagnosed within 2 or 4 years from blood draw.

We examined the association between *LEPR* SNPs and pancreatic cancer risk by modeling each genotype as number of copies of the minor allele (additive model) using conditional logistic regression. We used R software (version R.3.2.2.) to calculate the corrected *P*-value by taking into account the total number of comparisons, as well as correlations between 32 SNPs(26). We used HaploReg v4.1 to explore the noncoding functional characteristics of identified and highly correlated SNPs (r^2 >0.6 in 1000G CEU data). Statistical analyses were performed with SAS 9.1 (SAS Institute, Cary, North Carolina), and all *P* values are two sided.

RESULTS

The median time between blood collection and cancer diagnosis was 7.1 years among cases. Among controls, median plasma leptin was 20.8 μg/mL for women and 6.7 μg/mL for men. Leptin levels were comparable across studies for men (HPFS and PHS) and for women (NHS, WHI, and WHS) (Supplementary Table 2). Individuals with higher leptin levels had higher BMI, plasma C-peptide, and prevalence of diabetes (Table 1). After adjusting for age, fasting status and cohort, Spearman correlation coefficients for plasma leptin and BMI were 0.50 (P<0.0001) among men and 0.73 (P<0.0001) among women (Figure 1) (Supplementary table 3), similar to those reported in other populations (27,28).

We observed a positive association between plasma leptin and pancreatic cancer risk among men, but not among women ($P_{heterogeneity}$=0.02; Table 2). In the base model conditioned on matching factors, compared to the bottom quintile, men in the top quintile had an OR of 2.77 (1.37-5.61), (P_{trend}=0.01; Table 2). In comparison, women in the top quintile had an OR of 1.27 (0.84-1.91) (P_{trend}=0.64; Table 2). Further adjustment for race, multivitamin use, plasma 25(OH)D, history of diabetes, BMI, physical activity, plasma C-peptide, and plasma adiponectin yielded similar results (Table 2). Similar associations were observed in sensitivity analyses when we excluded cases with diabetes or cases diagnosed within 2 or 4 years of blood collection and their matched controls (Supplementary Table 4).

Spline curves were consistent with log-linear associations ($P_{nonlinear}$=0.81 for men; $P_{nonlinear}$=0.14 for women). Therefore, in subsequent meta-analyses and subgroup analyses, we modeled leptin as a continuous variable. The multivariate ORs for an increment of 5 ng/mL in plasma leptin were 1.25 (1.02-1.54) for men and 0.98 (0.93-1.03) for women (Supplementary Table 5). ORs were similar within the two male and the three female cohorts (Figure 2; $P_{heterogeneity}$=0.88 for HPFS and PHS, and $P_{heterogeneity}$=0.35 for NHS, WHI, and WHS). In

stratified analyses, no statistically significant effect modification was observed (Supplementary Table 5).

Several SNPs at the *LEPR* gene were associated with pancreatic cancer risk among women to $P<0.05$ in an additive genetic model (Table 3, Supplementary Figure 1). After adjusting for multiple comparisons, rs10493380 located intronic to *LEPR* remained statistically significantly associated with increased risk of pancreatic cancer (OR per minor allele=1.54; 95% CI=1.18-2.02, multiple hypothesis-corrected $P=0.03$; Table 3). This association was consistent across the three female cohorts ($P_{heterogeneity}=0.28$). The association for rs10493380 was not statistically significant among men (OR=1.19, 95% CI=0.79-1.78, multiple hypothesis-corrected $P=1.00$; Supplementary Table 6). Analysis of rs10493380 and highly correlated SNPs using HaploReg identified multiple transcription factor binding altered by these SNPs (Supplementary Table 7). Furthermore, in a blood eQTL database (29), rs10493380 (index SNP) and rs3790429 (in high LD with rs10493380, $r^2=0.90$ in 1000G EUR) were found to have *cis* eQTL effects on *LEPR* gene expression. No statistically significant association was identified between SNPs at the *LEPR* gene and plasma leptin levels among controls (Supplementary Table 8).

DISCUSSION

As seen in prior studies (23), plasma leptin levels were higher in our female compared with male control subjects, with a median level approximately three times higher in women versus men. Interestingly, higher prediagnostic plasma leptin was associated with an increased risk of pancreatic cancer in men, with no increase in risk was observed in women. This positive association among men was independent of other known risk factors for pancreatic cancer, including characteristics and plasma markers associated with obesity and insulin resistance. Furthermore, the association was highly consistent across two cohorts with male participants (positive association in HPFS and PHS) and three cohorts with female participants (no association in NHS, WHI, and WHS). In contrast, single nucleotide variants at the leptin receptor (*LEPR*) gene were associated with pancreatic cancer risk only in women, but not in men. This association was consistent across the three female cohorts. Notably, the most statistically significant SNP (rs10493380) and highly correlated variants at *LEPR* may alter leptin receptor gene expression based on bioinformatic analyses. In aggregate, these data support the importance of adipokines and adipokine signaling in pancreatic cancer risk in men and women, even though baseline circulating leptin levels differ greatly by gender.

A previous nested case-control study evaluated the association of prediagnostic plasma leptin with risk of pancreatic cancer (30). In this pooled analysis of three cohorts, plasma leptin was not associated with risk of pancreatic cancer during the first 10 years of follow-up, using gender-specific quintiles of plasma leptin. However, a statistically significant positive association was observed among men (OR, 2.94; 95% CI, 1.64-6.46; P_{trend}=0.001; comparing extreme quintiles) with longer follow-up time (≥10 years), while the association among women for this duration could not be evaluated due to small sample size. Similarly, in our cohorts, a

positive association of plasma leptin with pancreatic cancer risk was observed in men. However, in stratified analyses by time between blood collection and cancer diagnosis, statistically significant effect modification was not seen. This difference may be partly due to our *a priori* exclusion of cases with blood collected within 12 months of cancer diagnosis, the time period during which cancer-associated weight loss most commonly occurs (31). The stratified analyses in men were also limited by smaller sample sizes within strata. Three previous cross-sectional studies observed that plasma leptin levels were lower in pancreatic cancer patients compared to controls (32-34). However, in these retrospective studies, hypoleptinemia may have been due to the weight loss that is commonly experienced by patients with pancreatic cancer (5). Therefore, it is difficult to determine whether the observed low leptin levels contributed to pancreatic carcinogenesis or were a consequence of the cancer.

Several lines of evidence support a biological link between leptin and pancreatic carcinogenesis. Leptin plays a central role in the regulation of insulin sensitivity (35), and studies have demonstrated associations between hyperglycemia, insulin resistance, and future risk of pancreatic cancer (17,36-38). Therefore, one mechanism by which leptin may influence pancreatic cancer risk is through its modulation of insulin sensitivity (39). Leptin is synthesized by adipose tissue, and its concentration correlates with total body fat. Leptin may therefore act as a better marker for the relevant states of adiposity than BMI, which does not discriminate between fat and muscle mass (40). Target tissue effects of circulating leptin are not solely mediated centrally on cells within the hypothalamus, as leptin receptors are widely distributed in the body (7). Leptin receptors have been identified on the surface of tumor cells, including pluripotent cells thought to function as tumor initiating cells (41). Therefore, leptin signaling may have an important direct role in promoting tumor initiation and growth, independent of its

role in insulin sensitivity (42). As a consequence of this potential direct effect, inhibitors of the leptin receptor are being explored as novel therapeutics to inhibit tumor growth in patients (43).

We observed a positive association between plasma leptin and pancreatic cancer risk only in men. Interestingly, leptin levels are considerably lower in men than in women, even for the same age and body-mass index (44). Although the underlying reasons for this difference in circulating leptin are unclear, sex-differences in reproductive hormones and body fat distribution have been proposed as possible etiologies. Particularly, women tend to have higher total and subcutaneous fat, while men have a greater percentage of visceral fat, which may influence circulating levels of leptin (40). Given the substantially lower leptin levels and differing metabolic environment in men, the actions of leptin may be sex-specific, with implications for disease development. Alternatively, physiologic differences related to circulating leptin may be detectable only when compared in the lower ranges of circulating leptin, which are seen predominantly in men. Notably, a number of prospective cohort studies have examined the association of prediagnostic plasma leptin and the subsequent diabetes risk. Similar to the current study of pancreatic cancer, most of these studies demonstrated positive associations of leptin and diabetes in men, but not in women (45). Of note, crosstalk between estrogen and leptin signaling has been shown previously (46). Other than modulating synthesis of leptin (47), and leptin receptor (48), estrogen receptor alpha enhances leptin-induced activation of downstream signaling pathways, including the JAK/STAT pathway (46). Alternatively, the different association between leptin levels and pancreatic cancer risk between genders in our study could be due to chance, and these findings should be confirmed in future studies.

The current study has several strengths. The prospective design and exclusion of cases diagnosed within 1 year of blood collection reduced the potential impact of reverse causation on

our results. Furthermore, similar associations were observed when we excluded cases diagnosed within 2 or 4 years of blood collection. Leptin was measured in a single laboratory as a single batch, with low coefficients of variance for quality control samples. In our analyses, we adjusted for BMI, physical activity, and other biomarkers related to insulin resistance, including C-peptide and adiponectin, to rigorously control for confounding. We evaluated not only circulating leptin levels, but also genetic variants in the leptin receptor, which may affect signal transduction after ligand binding. Additional strengths included a large sample size, long follow-up period, and inclusion of men and women.

Plasma leptin was measured at only one point in time, so leptin levels may not fully reflect long-term plasma concentrations. However, leptin levels are relatively stable over time in healthy subjects; repeated plasma leptin measurements 1-year apart demonstrated a high intra-class correlation coefficient of 0.74 (21). We cannot rule out that residual confounding by adiposity not captured by BMI may be present; however, adjustment for plasma adiponectin, 25(OH)D and C-peptide as markers of adiposity and insulin resistance did not materially alter our results. We identified an association of rs10493380 with pancreatic cancer risk in women. This SNP was not identified as genome-wide significant in two recent pancreatic cancer genome-wide association studies (49,50). However, these studies were required to meet a stringent multiple-hypothesis testing threshold for statistical significance due to testing of >500,000 SNPs, included approximately 60% men, and included a majority of patients from tertiary center cases-control studies. Finally, our study population consisted primarily of White participants and further studies are required to evaluate circulating leptin in participants of different race/ethnicity.

In conclusion, we identified a positive association between prediagnostic circulating leptin levels and pancreatic cancer risk in men, independent of obesity and other risk factors of pancreatic cancer. Although this association was not observed in women, single nucleotide variants in the leptin receptor were associated with pancreatic cancer risk in women, and bioinformatic analyses suggested differences in leptin receptor expression with these variants. Our data provide additional evidence for a biological link between obesity, insulin resistance, and pancreatic cancer risk, specifically focusing attention on adipokines and adipokine signaling in pancreatic cancer development.

Acknowledgements:

The authors would like to thank the participants and staff of the Health Professionals Follow-up Study, Nurses' Health Study, Physicians' Health Study, Women's Health Initiative and Women's Health Study, for their valuable contributions as well as the following state cancer registries for their help: AL, AZ, AR, CA, CO, CT, DE, FL, GA, ID, IL, IN, IA, KY, LA, ME, MD, MA, MI, NE, NH, NJ, NY, NC, ND, OH, OK, OR, PA, RI, SC, TN, TX, VA, WA, WY. The authors assume full responsibility for analyses and interpretation of these data.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016;66(1):7-30.
2. Li D, Morris JS, Liu J, Hassan MM, Day RS, Bondy ML, et al. Body mass index and risk, age of onset, and survival in patients with pancreatic cancer. JAMA 2009;301(24):2553-62.
3. Michaud DS, Giovannucci E, Willett WC, Colditz GA, Stampfer MJ, Fuchs CS. Physical activity, obesity, height, and the risk of pancreatic cancer. JAMA 2001;286(8):921-9.
4. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature 1994;372(6505):425-32.
5. Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, et al. Serum immunoreactive-leptin concentrations in normal-weight and obese humans. N Engl J Med 1996;334(5):292-5.
6. Somasundar P, McFadden DW, Hileman SM, Vona-Davis L. Leptin is a growth factor in cancer. J Surg Res 2004;116(2):337-49.
7. Baskin DG, Blevins JE, Schwartz MW. How the brain regulates food intake and body weight: the role of leptin. J Pediatr Endocrinol Metab 2001;14 Suppl 6:1417-29.
8. Morioka T, Asilmaz E, Hu J, Dishinger JF, Kurpad AJ, Elias CF, et al. Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. J Clin Invest 2007;117(10):2860-8.
9. Garofalo C, Surmacz E. Leptin and cancer. J Cell Physiol 2006;207(1):12-22.
10. Giovannucci E, Ascherio A, Rimm EB, Colditz GA, Stampfer MJ, Willett WC. Physical activity, obesity, and risk for colon cancer and adenoma in men. Ann Intern Med 1995;122(5):327-34.
11. Colditz GA, Hankinson SE. The Nurses' Health Study: lifestyle and health among women. Nat Rev Cancer 2005;5(5):388-96.
12. Anderson GL, Manson J, Wallace R, Lund B, Hall D, Davis S, et al. Implementation of the Women's Health Initiative study design. Ann Epidemiol 2003;13(9 Suppl):S5-17.
13. Cook NR, Lee IM, Gaziano JM, Gordon D, Ridker PM, Manson JE, et al. Low-dose aspirin in the primary prevention of cancer: the Women's Health Study: a randomized controlled trial. JAMA 2005;294(1):47-55.
14. Gann PH, Ma J, Giovannucci E, Willett W, Sacks FM, Hennekens CH, et al. Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis. Cancer Res 1999;59(6):1225-30.
15. Hankinson SE, Willett WC, Manson JE, Hunter DJ, Colditz GA, Stampfer MJ, et al. Alcohol, height, and adiposity in relation to estrogen and prolactin levels in postmenopausal women. J Natl Cancer Inst 1995;87(17):1297-302.
16. Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;286(3):327-34.
17. Bao Y, Giovannucci EL, Kraft P, Stampfer MJ, Ogino S, Ma J, et al. A prospective study of plasma adiponectin and pancreatic cancer risk in five US cohorts. J Natl Cancer Inst 2013;105(2):95-103.
18. Marzi C, Albrecht E, Hysi PG, Lagou V, Waldenberger M, Tonjes A, et al. Genome-wide association study identifies two novel regions at 11p15.5-p13 and 1p31 with major impact on acute-phase serum amyloid A. PLoS Genet 2010;6(11):e1001213.

19. Sun Q, Cornelis MC, Kraft P, Qi L, van Dam RM, Girman CJ, et al. Genome-wide association study identifies polymorphisms in LEPR as determinants of plasma soluble leptin receptor levels. Hum Mol Genet 2010;19(9):1846-55.
20. Dehghan A, Dupuis J, Barbalic M, Bis JC, Eiriksdottir G, Lu C, et al. Meta-analysis of genome-wide association studies in >80 000 subjects identifies multiple loci for C-reactive protein levels. Circulation 2011;123(7):731-8.
21. Elliott P, Chambers JC, Zhang W, Clarke R, Hopewell JC, Peden JF, et al. Genetic Loci associated with C-reactive protein levels and risk of coronary heart disease. JAMA 2009;302(1):37-48.
22. Wu Y, McDade TW, Kuzawa CW, Borja J, Li Y, Adair LS, et al. Genome-wide association with C-reactive protein levels in CLHNS: evidence for the CRP and HNF1A loci and their interaction with exposure to a pathogenic environment. Inflammation 2012;35(2):574-83.
23. Havel PJ, Kasim-Karakas S, Dubuc GR, Mueller W, Phinney SD. Gender differences in plasma leptin concentrations. Nat Med 1996;2(9):949-50.
24. DerSimonian R, Laird N. Meta-analysis in clinical trials. Control Clin Trials 1986;7(3):177-88.
25. Durrleman S, Simon R. Flexible regression models with cubic splines. Stat Med 1989;8(5):551-61.
26. Conneely KN, Boehnke M. So many correlated tests, so little time! Rapid adjustment of P values for multiple correlated tests. Am J Hum Genet 2007;81(6):1158-68.
27. Sun Q, van Dam RM, Meigs JB, Franco OH, Mantzoros CS, Hu FB. Leptin and soluble leptin receptor levels in plasma and risk of type 2 diabetes in U.S. women: a prospective study. Diabetes 2010;59(3):611-8.
28. Li H, Stampfer MJ, Mucci L, Rifai N, Qiu W, Kurth T, et al. A 25-year prospective study of plasma adiponectin and leptin concentrations and prostate cancer risk and survival. Clin Chem 2010;56(1):34-43.
29. Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, et al. Systematic identification of trans eQTLs as putative drivers of known disease associations. Nat Genet 2013;45(10):1238-43.
30. Stolzenberg-Solomon RZ, Newton CC, Silverman DT, Pollak M, Nogueira LM, Weinstein SJ, et al. Circulating Leptin and Risk of Pancreatic Cancer: A Pooled Analysis From 3 Cohorts. Am J Epidemiol 2015;182(3):187-97.
31. Pannala R, Leibson CL, Rabe KG, Timmons LJ, Ransom J, de Andrade M, et al. Temporal association of changes in fasting blood glucose and body mass index with diagnosis of pancreatic cancer. Am J Gastroenterol 2009;104(9):2318-25.
32. Pezzilli R, Barassi A, Corsi MM, Morselli-Labate AM, Campana D, Casadei R, et al. Serum leptin, but not adiponectin and receptor for advanced glycation end products, is able to distinguish autoimmune pancreatitis from both chronic pancreatitis and pancreatic neoplasms. Scand J Gastroenterol 2010;45(1):93-9.
33. Dalamaga M, Migdalis I, Fargnoli JL, Papadavid E, Bloom E, Mitsiades N, et al. Pancreatic cancer expresses adiponectin receptors and is associated with hypoleptinemia and hyperadiponectinemia: a case-control study. Cancer Causes Control 2009;20(5):625-33.

34. Brown DR, Berkowitz DE, Breslow MJ. Weight loss is not associated with hyperleptinemia in humans with pancreatic cancer. J Clin Endocrinol Metab 2001;86(1):162-6.
35. Tilg H, Moschen AR. Adipocytokines: mediators linking adipose tissue, inflammation and immunity. Nat Rev Immunol 2006;6(10):772-83.
36. Gapstur SM, Gann PH, Lowe W, Liu K, Colangelo L, Dyer A. Abnormal glucose metabolism and pancreatic cancer mortality. JAMA 2000;283(19):2552-8.
37. Stolzenberg-Solomon RZ, Graubard BI, Chari S, Limburg P, Taylor PR, Virtamo J, et al. Insulin, glucose, insulin resistance, and pancreatic cancer in male smokers. JAMA 2005;294(22):2872-8.
38. Wolpin BM, Bao Y, Qian ZR, Wu C, Kraft P, Ogino S, et al. Hyperglycemia, insulin resistance, impaired pancreatic beta-cell function, and risk of pancreatic cancer. J Natl Cancer Inst 2013;105(14):1027-35.
39. Giovannucci E, Michaud D. The role of obesity and related metabolic disturbances in cancers of the colon, prostate, and pancreas. Gastroenterology 2007;132(6):2208-25.
40. Rosenbaum M, Nicolson M, Hirsch J, Heymsfield SB, Gallagher D, Chu F, et al. Effects of gender, body composition, and menopause on plasma concentrations of leptin. J Clin Endocrinol Metab 1996;81(9):3424-7.
41. Feldman DE, Chen C, Punj V, Tsukamoto H, Machida K. Pluripotency factor-mediated expression of the leptin receptor (OB-R) links obesity to oncogenesis through tumor-initiating stem cells. Proc Natl Acad Sci U S A 2012;109(3):829-34.
42. Khandekar MJ, Cohen P, Spiegelman BM. Molecular mechanisms of cancer development in obesity. Nat Rev Cancer 2011;11(12):886-95.
43. Surmacz E. Leptin and adiponectin: emerging therapeutic targets in breast cancer. J Mammary Gland Biol Neoplasia 2013;18(3-4):321-32.
44. Saad MF, Damani S, Gingerich RL, Riad-Gabriel MG, Khan A, Boyadjian R, et al. Sexual dimorphism in plasma leptin concentration. J Clin Endocrinol Metab 1997;82(2):579-84.
45. Chen GC, Qin LQ, Ye JK. Leptin levels and risk of type 2 diabetes: gender-specific meta-analysis. Obes Rev 2014;15(2):134-42.
46. Schmidt S, Monk JM, Robinson LE, Mourtzakis M. The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: potential effects of exercise. Obes Rev 2015;16(6):473-87.
47. Machinal F, Dieudonne MN, Leneveu MC, Pecquery R, Giudicelli Y. In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones. Endocrinology 1999;140(4):1567-74.
48. Bennett PA, Lindell K, Wilson C, Carlsson LM, Carlsson B, Robinson IC. Cyclical variations in the abundance of leptin receptors, but not in circulating leptin, correlate with NPY expression during the oestrous cycle. Neuroendocrinology 1999;69(6):417-23.
49. Childs EJ, Mocci E, Campa D, Bracci PM, Gallinger S, Goggins M, et al. Common variation at 2p13.3, 3q29, 7p13 and 17q25.1 associated with susceptibility to pancreatic cancer. Nat Genet 2015;47(8):911-6.
50. Wolpin BM, Rizzato C, Kraft P, Kooperberg C, Petersen GM, Wang Z, et al. Genome-wide association study identifies multiple susceptibility loci for pancreatic cancer. Nat Genet 2014;46(9):994-1000.

Table 1. Age- and study-standardized baseline characteristics according to plasma leptin levels among controls

	Quintiles of plasma leptin				
Characteristic*	**1**	**2**	**3**	**4**	**5**
Men					
Plasma leptin level, ng/mL	≤3.0	3.1-4.8	4.9-7.8	7.9-11.7	≥11.8
No. of controls	69	71	71	72	70
Age at blood draw, years	60.7 (10.1)	60.1 (8.9)	60.2 (10.0)	60.8 (8.9)	61.0 (9.1)
Race, %					
White	94.0	93.2	85.5	93.7	91.3
Black	0	1.0	0	0	0
Other	6.0	5.9	14.5	6.3	8.7
Body-mass index, kg/m^2	23.4 (2.6)	24.7 (2.1)	24.8 (2.3)	26.0 (2.5)	27.6 (3.6)
Physical activity, MET-hr/week	34.6 (34.9)	29.1 (31.6)	30.6 (48.6)	27.2 (34.6)	18.1 (22.6)
Cigarette smoking, %					
Never	37.0	36.9	37.3	40.9	33.6
Past	52.9	50.4	40.7	41.1	47.8
Current	10.2	12.7	22.0	18.1	18.6
Missing	0	0	0	0	0
History of diabetes mellitus, %	1.0	2.5	2.2	1.6	7.3
Regular multivitamin use, %	37.8	37.1	36.0	30.2	30.2
Plasma 25(OH)D, nmol/L	76.9 (28.9)	74.5 (32.0)	72.3 (20.7)	70.2 (20.9)	66.3 (19.5)
Plasma C-peptide levels, ng/mL	1.8 (1.1)	2.2 (1.4)	2.5 (1.6)	2.8 (3.4)	3.1 (2.9)
Plasma adiponectin levels, µg/mL	6.0 (3.2)	5.9 (2.8)	6.0 (3.3)	5.8 (3.2)	5.7 (4.1)
Women					
Plasma leptin level, ng/mL	≤9.5	9.6-17.7	17.8-24.8	24.9-37.7	≥37.8
No. of controls	148	146	150	148	149
Age at blood draw, years	63.3 (8.3)	63.2 (7.9)	64.3 (8.3)	63.1 (7.3)	63.1 (7.8)
Race, %					
White	89.2	95.8	94.9	92.1	93.9
Black	2.4	0.6	0.9	4.6	4.1
Other	8.4	3.6	4.2	3.4	2.1
Body-mass index, kg/m^2	21.8 (2.6)	24.2 (3.9)	25.7 (3.7)	27.4 (3.2)	31.3 (5.2)
Physical activity, MET-hr/week	21.7 (20.4)	17.4 (18.2)	15.3 (16.8)	13.0 (13.8)	12.0 (11.8)
Cigarette smoking, %					
Never	41.1	43.6	52.9	37.7	50.7
Past	42.8	44.3	38.5	48.7	42.4

Current	14.7	10.7	8.1	13.0	6.0
Missing	1.4	1.5	0.5	0.7	1.0
History of diabetes mellitus, %	1.7	0	3.9	3.9	7.7
Regular multivitamin use, %	47.3	42.6	45.2	43.6	36.3
Plasma 25(OH)D, nmol/L	70.0 (25.3)	65.7 (21.1)	62.7 (20.3)	57.4 (32.4)	54.6 (18.3)
Plasma C-peptide levels, ng/mL	1.3 (0.6)	1.6 (0.7)	2.0 (1.2)	2.2 (1.0)	2.5 (1.2)
Plasma adiponectin levels, µg/mL	11.1 (6.0)	9.6 (4.8)	8.7 (4.7)	7.7 (4.7)	8.0 (4.6)

* Mean (standard deviation) for all continuous variables

Abbreviations: 25(OH)D, 25-hydroxyvitamin D ; MET-hr, metabolic equivalent of task-hour

Table 2. Odds ratios (ORs) and 95% confidence intervals (CIs) for pancreatic cancer according to quintiles of plasma leptin

	Quintiles of plasma leptin					P_{trend}*
	1	**2**	**3**	**4**	**5**	
Men						
Leptin levels, ng/mL						
Range	≤3.0	3.1-4.8	4.9-7.8	7.9-11.7	≥11.8	
Median	2.2	4.0	6.7	9.2	15.3	
No. of cases	17	26	32	27	42	
No. of controls	69	71	71	72	70	
Base model[1]	1.0	1.57 (0.78-3.16)	1.90 (0.95-3.80)	1.61 (0.80-3.24)	2.77 (1.37-5.61)	0.01
Adjusted model I[2]	1.0	1.48 (0.72-3.05)	1.80 (0.89-3.64)	1.59 (0.78-3.25)	2.55 (1.23-5.27)	0.02
Adjusted model II[3]	1.0	1.48 (0.72-3.05)	1.80 (0.88-3.67)	1.60 (0.77-3.33)	2.54 (1.13-5.72)	0.03
Adjusted model III[4]	1.0	1.68 (0.79-3.54)	2.03 (0.96-4.29)	1.84 (0.86-3.95)	3.02 (1.27-7.16)	0.02
Women						
Leptin levels, ng/mL						
Range	≤9.5	9.6-17.7	17.8-24.8	24.9-37.7	≥37.8	
Median	6.2	14.0	20.8	30.6	51.5	
No. of cases	60	80	45	60	81	
No. of controls	148	146	150	148	149	
Base model[1]	1.0	1.36 (0.91-2.03)	0.76 (0.49-1.19)	0.98 (0.64-1.50)	1.27 (0.84-1.91)	0.64
Adjusted model I[2]	1.0	1.36 (0.91-2.03)	0.74 (0.48-1.16)	0.95 (0.61-1.47)	1.19 (0.78-1.82)	0.90
Adjusted model II[3]	1.0	1.27 (0.84-1.92)	0.68 (0.43-1.09)	0.82 (0.50-1.33)	0.94 (0.55-1.62)	0.37
Adjusted model III[4]	1.0	1.26 (0.82-1.93)	0.63 (0.38-1.03)	0.72 (0.43-1.21)	0.84 (0.46-1.51)	0.21

*P_{trend} values were calculated by the Wald test of a score variable that contained median values of quintiles.

[1]ORs and 95% CI were estimated by conditional logistic regression conditioned on the matching factors including year of birth, prospective cohort (HPFS, NHS, PHS, WHI, WHS), smoking status (never, past, current), fasting status (fasting, non-fasting), and month/year of blood draw.

[2] Further adjusted for race (White, Black, other), history of diabetes mellitus (yes, no), current multivitamin use (yes, no), and plasma 25(OH)D (continuous).

[3]Further adjusted for BMI (continuous) and physical activity (continuous).

[4]Further adjusted for plasma C-peptide (continuous) and plasma adiponectin (quartiles).

Abbreviations: 25(OH)D, 25-hydroxyvitamin D ; BMI, body mass index; HPFS, Health Professionals Follow-up Study; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study

Table 3. Association between SNPs in the leptin receptor (*LEPR*) gene and risk of pancreatic cancer among women

SNP	Minor allele	Controls		Cases		Additive Model		
		N	MAF (%)	N	MAF (%)	OR[a] (95% CI)	Raw *P*-value	Corrected *P*-value
rs10493380	C	646	0.16	272	0.21	1.54 (1.18-2.02)	0.001	0.032
rs3790424	G	655	0.31	280	0.24	0.69 (0.55-0.89)	0.003	0.069
rs6673324	G	650	0.48	277	0.55	1.38 (1.11-1.71)	0.003	0.072
rs2154381	G	653	0.34	281	0.27	0.72 (0.57-0.90)	0.004	0.088
rs6662904	A	648	0.42	278	0.48	1.32 (1.07-1.62)	0.009	0.170
rs9436746	A	653	0.45	280	0.39	0.75 (0.61-0.93)	0.010	0.180
rs9436747	A	653	0.42	278	0.35	0.75 (0.60-0.93)	0.010	0.184
rs11801408	A	657	0.18	283	0.23	1.36 (1.06-1.75)	0.014	0.243
rs41459646	C	654	0.16	279	0.20	1.36 (1.05-1.77)	0.021	0.329
rs2767485	G	650	0.18	280	0.22	1.27 (0.99-1.63)	0.059	0.647
rs9436301	G	650	0.23	280	0.27	1.25 (0.99-1.58)	0.063	0.672
rs7524834	G	659	0.42	282	0.46	1.21 (0.98-1.49)	0.074	0.724
rs12025906	G	657	0.21	280	0.18	0.79 (0.61-1.03)	0.084	0.770
rs9436748	A	654	0.39	278	0.43	1.18 (0.95-1.46)	0.134	0.901
rs10128072	C	650	0.15	277	0.18	1.19 (0.90-1.58)	0.210	0.975
rs7602	A	655	0.20	279	0.23	1.17 (0.91-1.50)	0.222	0.981
rs1887285	G	657	0.09	277	0.11	1.23 (0.88-1.72)	0.233	0.984
rs3828033	A	647	0.37	276	0.35	0.89 (0.72-1.10)	0.293	0.996
rs913199	A	652	0.45	279	0.49	1.09 (0.89-1.33)	0.420	1.000
rs1892534	A	653	0.39	278	0.41	1.08 (0.87-1.34)	0.474	1.000
rs3790431	G	651	0.20	279	0.19	0.91 (0.71-1.18)	0.482	1.000
rs3806318	G	654	0.26	279	0.28	1.08 (0.86-1.36)	0.519	1.000
rs2148683	G	655	0.47	282	0.49	1.06 (0.87-1.30)	0.537	1.000
rs2148682	G	655	0.35	280	0.37	1.05 (0.85-1.30)	0.649	1.000
rs12753193	G	659	0.39	279	0.40	1.05 (0.85-1.30)	0.663	1.000
rs4420065	A	651	0.39	279	0.40	1.04 (0.84-1.29)	0.691	1.000
rs4655537	A	654	0.36	275	0.37	1.04 (0.84-1.29)	0.736	1.000
rs6700896	A	654	0.39	280	0.40	1.03 (0.83-1.27)	0.776	1.000
rs11585329	A	655	0.16	279	0.14	0.97 (0.72-1.29)	0.817	1.000
rs9436737	G	650	0.14	274	0.14	1.02 (0.76-1.36)	0.900	1.000
rs3790436	C	653	0.45	277	0.46	1.01 (0.82-1.25)	0.920	1.000
rs17127601	G	649	0.13	278	0.13	1.01 (0.75-1.37)	0.926	1.000

[a] ORs and 95% CI were estimated using conditional logistic regression, conditioning on matching factors including year of birth, prospective cohort (HPFS, NHS, PHS, WHI, WHS) which also conditions on gender, smoking status (never, past, current), fasting status (fasting, non fasting), and month/year of blood draw, and adjusted for race (White, Black, other), history of diabetes mellitus (yes, no), current multivitamin use (yes, no), plasma 25(OH)D (continuous), BMI (continuous), physical activity (continuous), plasma C-peptide (continuous) and plasma adiponectin (quartiles).

Abbreviations: 25(OH)D, 25-hydroxyvitamin D ; BMI, body mass index; CI, confidence ratio ; HPFS, Health Professionals Follow-up Study; OR, odds ratio; MAF, minor allele frequency; NHS, Nurses' Health Study; PHS, Physicians' Health Study; SNP, single nucleotide polymorphism; WHI, Women's Health Initiative; WHS, Women's Health Study

Figure titles and legends

Figure 1.

Title:

Correlation between body-mass index and plasma leptin in men and women

Legend:

Scatterplot of body-mass index *vs.* plasma leptin for women (blue squares) and men (red squares). Trendline (line of best fit) is shown as full (men) or dashed line (women).

Abbreviations: BMI, body-mass index ; *P*-corr, *P* correlation

Figure 2.

Title:

Cohort-specific and meta-analysis of pooled odds ratios for pancreatic cancer according to plasma leptin levels (per 5 ng/mL increase in plasma leptin).

Legend:

Cohort-specific multivariate odds ratios conditioned on matching factors including year of birth, prospective cohort (HPFS, NHS, PHS, WHI, WHS), smoking status (never, past, current), fasting status (fasting, non-fasting), and month/year of blood draw, and adjusted for covariates including race (White, Black, other), history of diabetes mellitus (yes, no), current multivitamin use (yes, no), plasma 25(OH)D (continuous), body-mass index (continuous), physical activity (continuous), plasma C-peptide (continuous), and plasma adiponectin (quartiles). The pooled odds ratio is calculated by the DerSimonian and Laird random-effects model. The solid squares and horizontal lines correspond to the cohort-specific multivariate odds ratios and 95% confidence intervals, respectively. The area of the solid square reflects the cohort-specific weight (inverse of the variance). The open diamond represents the pooled multivariate odds ratio and 95% confidence interval. The solid vertical line indicates an odds ratio of 1.0.

Abbreviations: 25(OH)D, 25-hydroxyvitamin D ; CI, confidence ratio ; HPFS, Health Professionals Follow-up Study; OR, odds ratio; NHS, Nurses' Health Study; PHS, Physicians' Health Study; WHI, Women's Health Initiative; WHS, Women's Health Study

Figure 1

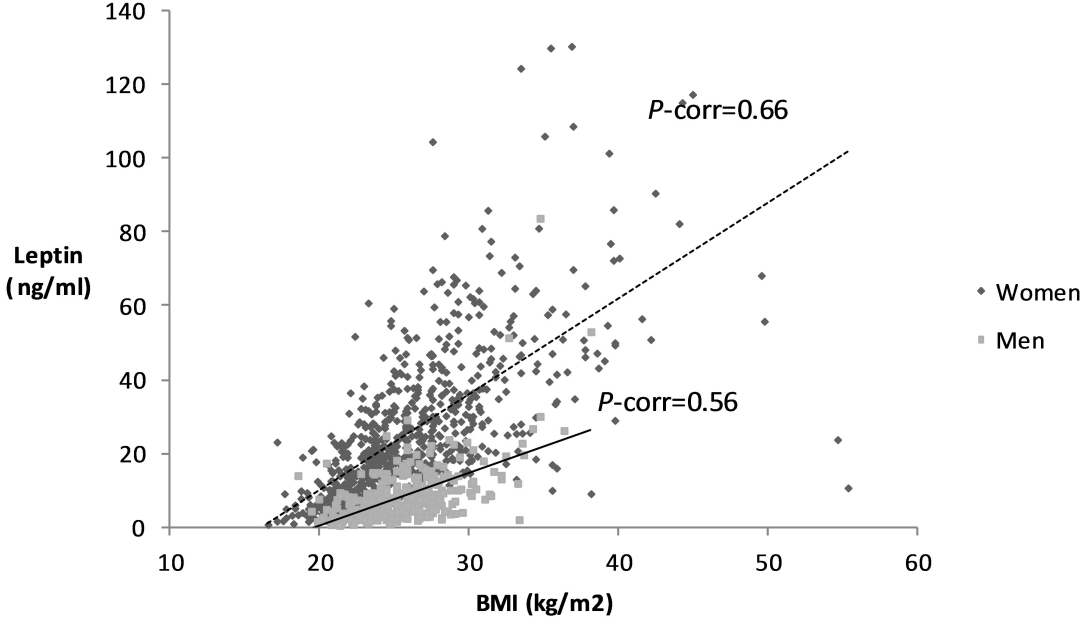
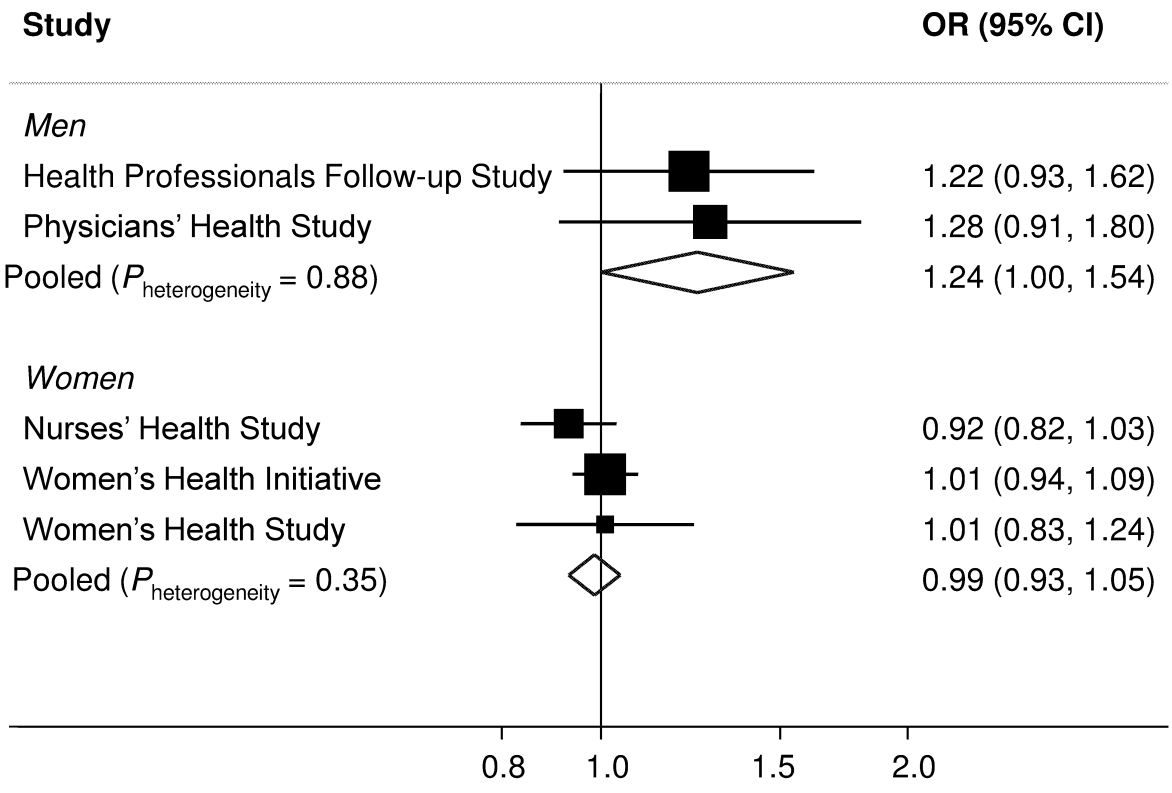

140
120
100
80
60
40
20
0
Leptin (ng/ml)
10
20
30
40
50
60
BMI (kg/m2)
P-corr=0.66
P-corr=0.56
Women
Men

Figure 2.


Study
OR (95% CI)
Men
Health Professionals Follow-up Study
1.22 (0.93, 1.62)
Physicians' Health Study
1.28 (0.91, 1.80)
Pooled ($P_{heterogeneity}$ = 0.88)
1.24 (1.00, 1.54)
Women
Nurses' Health Study
0.92 (0.82, 1.03)
Women's Health Initiative
1.01 (0.94, 1.09)
Women's Health Study
1.01 (0.83, 1.24)
Pooled ($P_{heterogeneity}$ = 0.35)
0.99 (0.93, 1.05)
0.8
1.0
1.5
2.0

REFERENCES AND NOTES

1. R. Majumdar, M. Sixt, C. A. Parent, *Curr. Opin. Cell Biol.* **30**, 33–40 (2014).
2. P. Roca-Cusachs, R. Sunyer, X. Trepat, *Curr. Opin. Cell Biol.* **25**, 543–549 (2013).
3. C.-M. Lo, H.-B. Wang, M. Dembo, Y. L. Wang, *Biophys. J.* **79**, 144–152 (2000).
4. B. C. Isenberg, P. A. Dimilla, M. Walker, S. Kim, J. Y. Wong, *Biophys. J.* **97**, 1313–1322 (2009).
5. M. Raab *et al.*, *J. Cell Biol.* **199**, 669–683 (2012).
6. S. V. Plotnikov, A. M. Pasapera, B. Sabass, C. M. Waterman, *Cell* **151**, 1513–1527 (2012).
7. T. Kawano, S. Kidoaki, *Biomaterials* **32**, 2725–2733 (2011).
8. J. R. Tse, A. J. Engler, *PLOS ONE* **6**, e15978 (2011).
9. L. G. Vincent, Y. S. Choi, B. Alonso-Latorre, J. C. del Álamo, A. J. Engler, *Biotechnol. J.* **8**, 472–484 (2013).
10. L. Bollmann *et al.*, *Front. Cell. Neurosci.* **9**, 363 (2015).
11. L. A. Flanagan, Y.-E. Ju, B. Marg, M. Osterfield, P. A. Janmey, *Neuroreport* **13**, 2411–2415 (2002).
12. F. Liu *et al.*, *J. Cell Biol.* **190**, 693–706 (2010).
13. T. A. Ulrich, E. M. de Juan Pardo, S. Kumar, *Cancer Res.* **69**, 4167–4174 (2009).
14. D. Cai *et al.*, *Cell* **157**, 1146–1159 (2014).
15. E. Donà *et al.*, *Nature* **503**, 285–289 (2013).
16. A. Haeger, K. Wolf, M. M. Zegers, P. Friedl, *Trends Cell Biol.* **25**, 556–566 (2015).
17. A. Bianco *et al.*, *Nature* **448**, 362–365 (2007).
18. B. A. Camley, J. Zimmermann, H. Levine, W.-J. Rappel, *Phys. Rev. Lett.* **116**, 098101 (2016).
19. G. Malet-Engra *et al.*, *Curr. Biol.* **25**, 242–250 (2015).
20. E. Theveneau *et al.*, *Dev. Cell* **19**, 39–53 (2010).
21. R. Sunyer, A. J. Jin, R. Nossal, D. L. Sackett, *PLOS ONE* **7**, e46107 (2012).
22. Materials and methods are available as supplementary materials on *Science* Online.
23. A. Elosegui-Artola *et al.*, *Nat. Mater.* **13**, 631–637 (2014).
24. D. T. Tambe *et al.*, *Nat. Mater.* **10**, 469–475 (2011).
25. E. Bazellières *et al.*, *Nat. Cell Biol.* **17**, 409–420 (2015).
26. C. E. Chan, D. J. Odde, *Science* **322**, 1687–1691 (2008).
27. A. Elosegui-Artola *et al.*, *Nat. Cell Biol.* **18**, 540–548 (2016).
28. J. Escribano, M. T. Sánchez, J. M. García-Aznar, *Comput. Part. Mech.* **1**, 117–130 (2014).
29. A. Berdahl, C. J. Torney, C. C. Ioannou, J. J. Faria, I. D. Couzin, *Science* **339**, 574–576 (2013).
30. P. M. Krafft, R. X. Hawkins, A. S. Pentland, N. D. Goodman, J. B. Tenenbaum, in *Proceedings of the 37th Conference of the Cognitive Science Society* (Cognitive Science Society, 2015).

ACKNOWLEDGMENTS

The authors acknowledge X. Serra-Picamal, V. Romaric, R. Alert, J. Cadademunt, and all members of the X.T. and P.R.-C. laboratories for stimulating discussions; N. Castro for technical assistance; and G. Charras, G. Scita, and E. Sahai for providing cells. This work was supported by the Spanish Ministry of Economy and Competitiveness (BFU2015-65074-P to X.T., BFU2011-23111 and BFU2014-52586-REDT to P.R.-C., DPI2015-64221-C2-1-R to J.M.G.-A., DPI2013-43727-R to J.J.M., PI14/00280 to D.N., RYC-2014-15559 to V.C., FPI BES-2013-063684 to J.E., IJCI-2014-19156 to A.E.-A., and IJCI-2014-19843 to A.L.), the Generalitat de Catalunya (2014-SGR-927), the European Research Council (CoG-616480 to X.T. and StG 306571 to J.M.G.-A.), Obra Social "La Caixa," Marie-Curie Action (CAFFORCE 328664 to A.L.), EMBO Long-Term Fellowship (EMBO ALTF 1235-2012 to A.L.), a Career Integration Grant within the seventh European Community Framework Programme (PCIG10-GA-2011-303848 to P.R.-C.), and Fundació la Marató de TV3 (project 20133330 to P.R.-C.).

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6304/1157/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S16
Tables S1 and S2
Movies S1 to S10
References (*31*–*43*)

22 March 2016; accepted 27 July 2016
10.1126/science.aaf7119

TUMOR METABOLISM

Tissue of origin dictates branched-chain amino acid metabolism in mutant *Kras*-driven cancers

Jared R. Mayers,[1,2]* Margaret E. Torrence,[1,2]* Laura V. Danai,[1] Thales Papagiannakopoulos,[1]† Shawn M. Davidson,[1,2] Matthew R. Bauer,[1] Allison N. Lau,[1] Brian W. Ji,[3] Purushottam D. Dixit,[3] Aaron M. Hosios,[1,2] Alexander Muir,[1] Christopher R. Chin,[1] Elizaveta Freinkman,[1,2,4,5,6] Tyler Jacks,[1,2,6] Brian M. Wolpin,[7] Dennis Vitkup,[3] Matthew G. Vander Heiden[1,2,5,7]‡

Tumor genetics guides patient selection for many new therapies, and cell culture studies have demonstrated that specific mutations can promote metabolic phenotypes. However, whether tissue context defines cancer dependence on specific metabolic pathways is unknown. *Kras* activation and *Trp53* deletion in the pancreas or the lung result in pancreatic ductal adenocarcinoma (PDAC) or non–small cell lung carcinoma (NSCLC), respectively, but despite the same initiating events, these tumors use branched-chain amino acids (BCAAs) differently. NSCLC tumors incorporate free BCAAs into tissue protein and use BCAAs as a nitrogen source, whereas PDAC tumors have decreased BCAA uptake. These differences are reflected in expression levels of BCAA catabolic enzymes in both mice and humans. Loss of Bcat1 and Bcat2, the enzymes responsible for BCAA use, impairs NSCLC tumor formation, but these enzymes are not required for PDAC tumor formation, arguing that tissue of origin is an important determinant of how cancers satisfy their metabolic requirements.

The development of new cancer therapeutics relies on underlying genetic features to identify sensitive patients (*1*). Mutations in both *KRAS* and *TP53* are common genetic events found in tumors arising from many tissues, and cancers with these mutations are often difficult to treat (*2*, *3*). These genetic events, as well as others associated with cancer, contribute to the metabolic changes that support biomass accumulation and cancer cell proliferation (*4*). Oncogenic *RAS* signaling increases glucose and glutamine consumption to support anabolic processes including nucleotide, lipid, and nonessential amino acid biosynthesis and can also drive extracellular protein and lipid scavenging (*5*). *TP53* mutations increase glucose consumption and glycolytic flux, whereas inactivation of *TP53* renders cancer cells more dependent on serine uptake and metabolism (*6*).

KRAS and *TP53* mutations are found in most human pancreatic tumors (*7*) and are also common in lung adenocarcinoma (*8*). What is known of how mutant *KRAS* or disruption of *TP53* affects cancer metabolism is based on cell culture studies in defined medium, although in vivo nutrient availability varies widely between tissues, and vasculature changes can limit nutrient access within tumors (*9*, *10*). The inability to model these differences in culture has therefore limited understanding of how tissue of origin influences tumor metabolism (*11*). Furthermore, environment can influence metabolic phenotypes in vitro (*12*–*14*), and metabolic dependencies in vivo can differ from those found in vitro (*15*). Metabolic differences between tumor types may also result from cell-autonomous effects, and tumor metabolic gene expression more closely resembles that of its tissue of origin than that of other tumors (*16*). The same oncogenic driver can also cause different metabolic phenotypes in lung and liver tumors (*17*). This raises the possibility that tumor type is a major determinant of some tumor metabolic dependencies in vivo.

Elevated plasma branched-chain amino acid (BCAA) levels are found in early pancreatic ductal adenocarcinoma (PDAC) but not in non–small cell lung carcinoma (NSCLC), even when the tumors are initiated by the same genetic events (*18*). To confirm that tumor tissue of origin influences whole-body BCAA metabolism, we used *LSL–Kras*$^{G12D/+}$;*Trp53*$^{flox/flox}$ (KP) mice. We crossed KP mice to mice harboring a *Cre-recombinase* allele driven by a *Pdx-1* promoter (KP$^{-/-}$C model) (*19*) or delivered viral *Cre* to the lungs of these mice (*20*) in order to generate models of PDAC and NSCLC, respectively. Consistent with prior reports (*18*), mice with early PDAC have increased levels of plasma BCAAs, whereas mice with early NSCLC exhibit decreased plasma BCAA levels (fig. S1, A to D). When cells derived from these tumors

[1]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [3]Center for Computational Biology and Bioinformatics and Department of Systems Biology, Columbia University, New York, NY 10027, USA. [4]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA. [5]Broad Institute, Seven Cambridge Center, Cambridge, MA 02142, USA. [6]Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. [7]Dana-Farber Cancer Institute, Boston, MA 02115, USA.
*These authors contributed equally to this work.
†Present address: School of Medicine, New York University, New York, NY 10016, USA. ‡Corresponding author. Email: mvh@mit.edu

are implanted subcutaneously into syngeneic hosts, tumors derived from PDAC cells did not affect plasma BCAA levels (fig. S1E) (*18*), whereas tumors derived from NSCLC cells led to decreased plasma BCAAs (fig. S1F). These results suggest that tumor formation from NSCLC cells can cause depletion of circulating BCAAs.

To trace tissue-specific differences in BCAA metabolism in animals with pancreatic or lung tumors, mice were fed an amino acid–defined diet in which 20% of leucine and valine were ^{13}C-labeled. All groups of mice exhibited similar levels of plasma ^{13}C-BCAA enrichment after 1 week of exposure to labeled diets (figs. S2, A and B). Whereas PDAC tumors contained slightly decreased free BCAAs relative to normal pancreas, NSCLC tumors displayed a significant increase in labeled free BCAAs compared with normal lung (Fig. 1A and fig. S2, C and D). These differences are not a reflection of different amino acid compositions of normal or tumor tissue in either the PDAC or NSCLC models (fig. S3). Because BCAAs are essential amino acids that animals cannot synthesize de novo (*21*), these results suggest that unlike PDAC tumors, NSCLC tumors display enhanced BCAA uptake.

BCAAs have several potential metabolic fates in tissues (Fig. 1B). They can be directly incorporated into protein or reversibly transaminated by BCAA transaminase (Bcat) to produce branched-chain α-ketoacids (BCKAs) and glutamate. BCKAs can regenerate BCAAs, be secreted, or be oxidatively decarboxylated by the branched-chain ketoacid dehydrogenase (Bckdh) complex to allow further oxidation of the carbon skeleton (*21*). In agreement with increased BCAA uptake in NSCLC tumors, lung tumors displayed increased labeled BCAA incorporation into protein compared with normal lung, whereas PDAC tumors incorporated less labeled BCAAs relative to normal pancreas (Fig. 1C and fig. S2, E and F). Analysis of metabolites derived from BCAA catabolism revealed that NSCLC tumors also had more labeled α-ketoisocaproate (KIC), the leucine-derived BCKA, whereas no change was observed in levels of this labeled metabolite in PDAC tumors (Fig. 1D and fig. S2G). No other differences in labeled BCAA catabolite levels were observed in NSCLC compared with normal tissues, but PDAC tumors showed decreased labeling of the tricarboxylic acid (TCA) cycle intermediate citrate relative to normal pancreas from labeled BCAAs (Fig. 1E and fig. S2, G to I). This is consistent with recent work demonstrating minimal catabolism of BCAAs to TCA intermediates in proliferating cells (*22*). We then explored whether excess KIC may be excreted by NSCLC tumors for further metabolism by other tissues, such as liver, which has limited Bcat but high Bckdh activity (*21*, *23*). Consistent with this hypothesis, we observed increased labeling of downstream leucine metabolites in the livers of mice with lung tumors (fig. S4). Taken together, these data suggest that BCAA uptake and transamination, but not their subsequent catabolism, may provide a benefit to NSCLC tumors, potentially by acting as a source of nitrogen.

To examine whether NSCLC tumors, but not PDAC tumors, use BCAAs as a source of nitrogen,

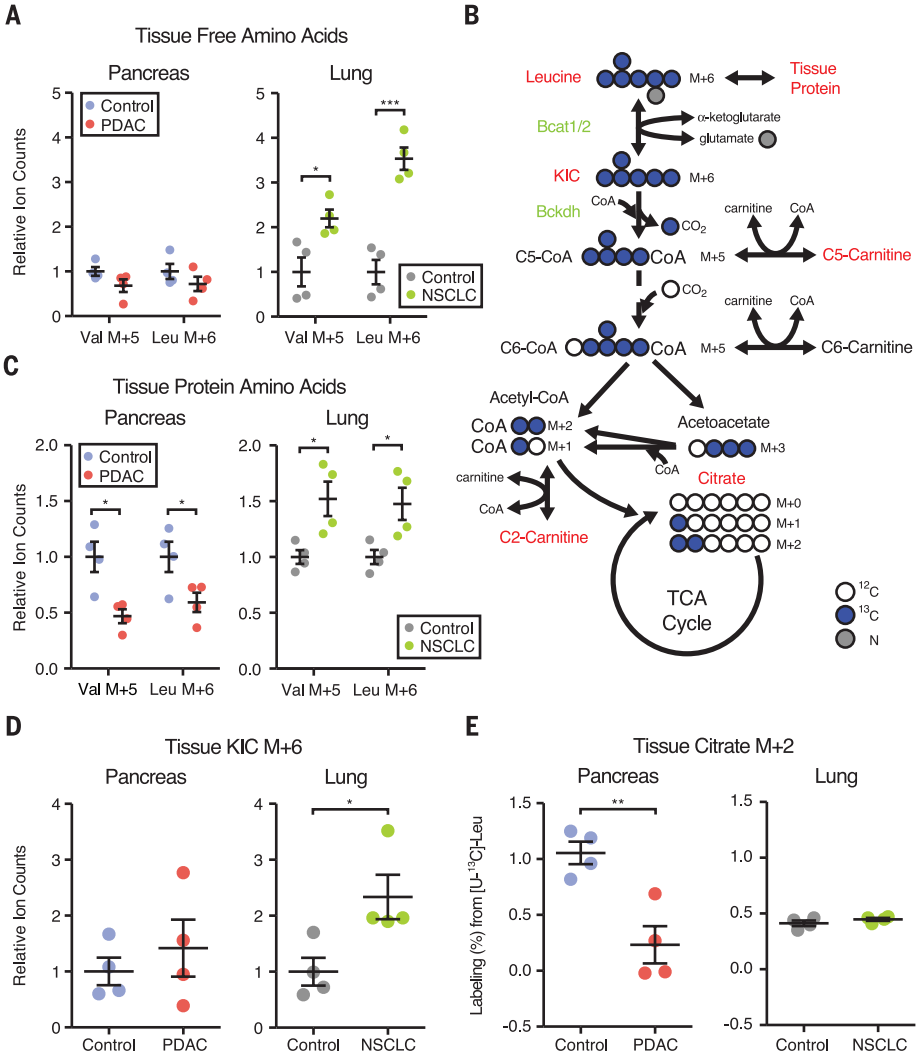


Fig. 1. Mice with NSCLC display increased BCAA uptake and metabolism. (**A** and **C** to **E**) Mice were fed ^{13}C-BCAA–containing diet for 7 days. (A) Relative ion counts by means of liquid chromatography–mass spectrometry (LC-MS) analysis of fully labeled, free BCAAs in tumors from PDAC and NSCLC mice and normal tissues from their respective control mice. Data are presented as mean ± SEM; n = 4 control and n = 4 PDAC; n = 4 control and n = 4 NSCLC. (**B**) Diagram of the leucine catabolic pathway. Red labels indicate metabolites measured with mass spectrometry. Blue circles indicate ^{13}C-labeled carbons. KIC, α-ketoisocaproate. (C) Relative ion counts by means of gas chromatography (GC)–MS analysis of fully labeled BCAAs from protein acid hydrolysates of tumors from PDAC and NSCLC mice and normal tissues from their respective control mice. Data are presented as mean ± SEM; n = 4 control and n = 4 PDAC; n = 4 control and n = 4 NSCLC. (D) Relative ion counts by means of LC-MS analysis of fully labeled KIC in tumors from PDAC and NSCLC mice and normal tissues from their respective control mice. Data are presented as mean ± SEM; n = 4 control and n = 4 PDAC; n = 4 control and n = 4 NSCLC. (E) Citrate M+2 labeling (%) from [U-^{13}C]-leucine by means of GC-MS analysis in tumors from PDAC and NSCLC mice and normal tissues from their respective control mice. Data are presented as mean ± SEM; n = 4 control and n = 4 PDAC; n = 4 control and n = 4 NSCLC. Two-tailed t test was used for all comparisons between two groups. *P < 0.05, **P < 0.01, ***P < 0.001.

we fed mice a modified amino acid diet in which 50% of leucine was labeled with ^{15}N, allowing the fate of leucine-derived nitrogen to be traced (Fig. 2A). In agreement with ^{13}C-tracing, mice with PDAC demonstrated no differences in free ^{15}N-labeled leucine in tumors compared with control pancreas (fig. S5A) and had less ^{15}N incorporation into other amino acids (fig. S5B). In contrast, increased levels of ^{15}N-leucine were found in NSCLC tumors compared with normal lung (fig. S5C) with decreased plasma enrichment of ^{15}N-leucine in mice with NSCLC tumors (Fig. 2B and fig. S5D). A relative increase in ^{15}N-labeling of nonessential amino acids, as well as valine and isoleucine, was observed in both the free and tissue-protein amino acid pools of NSCLC compared with control lung (Fig. 2C and fig. S5, C and E). Given the reduced plasma enrichment of

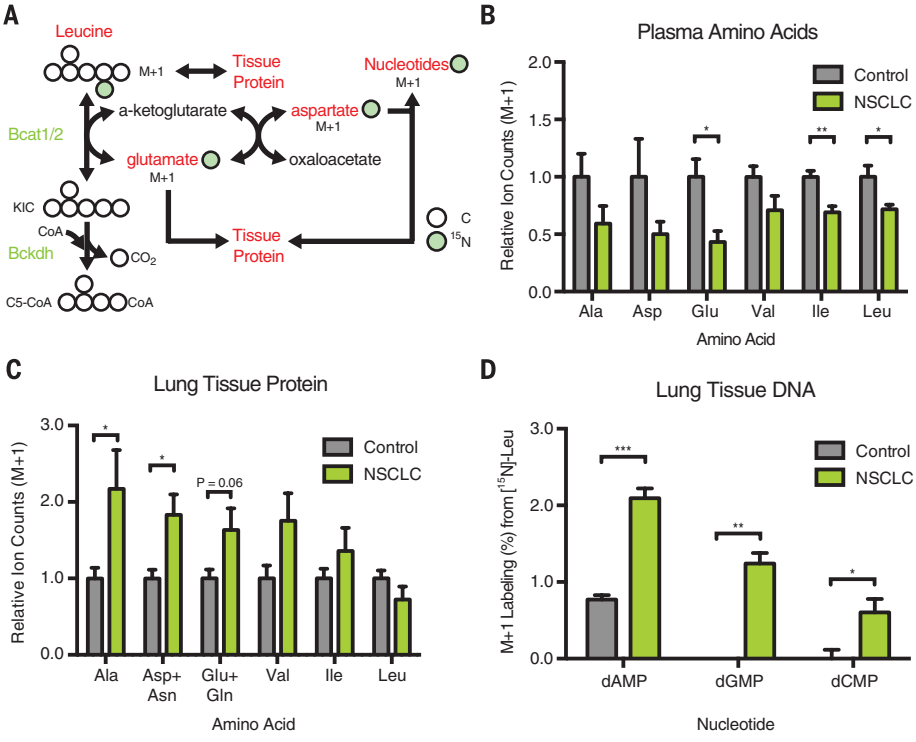


Fig. 2. BCAA-derived nitrogen supports nonessential amino acid and DNA synthesis in NSCLC tumors. (**A**) Diagram of leucine transamination by Bcat and nitrogen (green circles) fate after transamination. (**B** to **D**) NSCLC mice were fed ^{15}N-leucine containing diet for 6 days. (B) Relative ion counts by means of GC-MS analysis of M+1-labeled amino acids in plasma of control and NSCLC mice. Data are presented as mean ± SEM; n = 5 control and n = 6 NSCLC. (C) Relative ion counts by means of GC-MS analysis of M+1-labeled amino acids from protein acid hydrolysates of control mouse lung tissue and NSCLC mouse tumors. Data are presented as mean ± SEM; n = 6 control and n = 6 NSCLC. (D) M+1 labeling (%) from ^{15}N-leucine of deoxynucleic acids from nucleic acid digest of control mouse lung tissue and NSCLC mouse tumors. Data are presented as mean ± SEM; n = 6 control and n = 6 NSCLC. Two-tailed t test was used for all comparisons between two groups. *P < 0.05, **P < 0.01, ***P < 0.001.

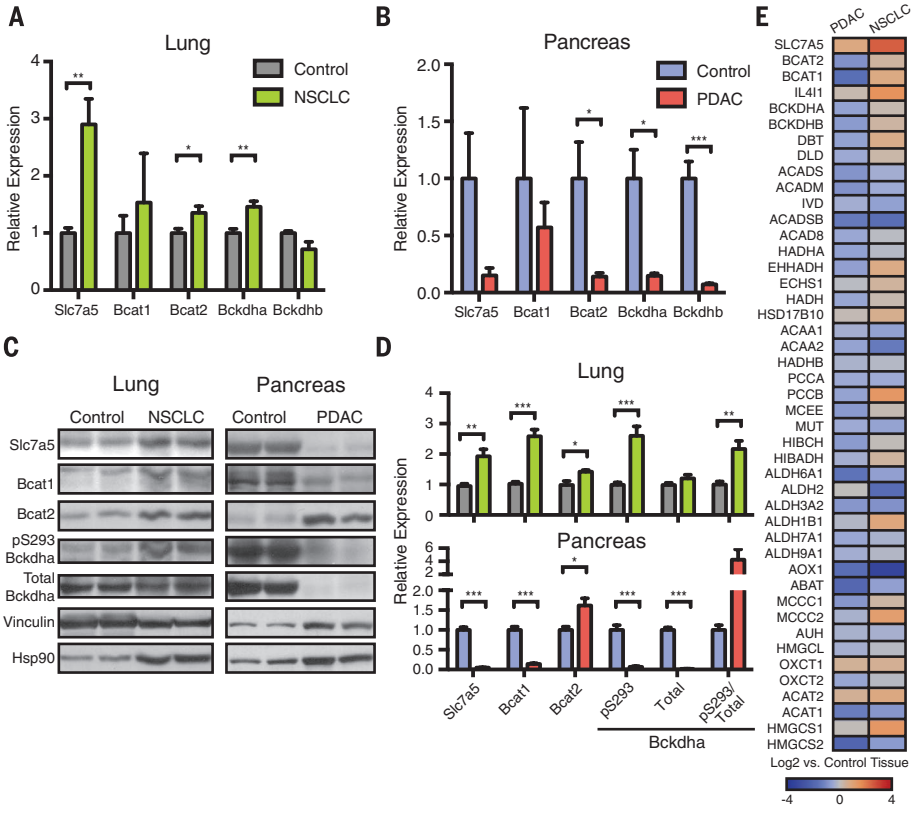


Fig. 3. Gene expression in both mouse and human tumors reflects tumor tissue–specific BCAA metabolism. (**A**) Relative expression of BCAA metabolic pathway genes in normal lung and NSCLC tumors from KP mice. Data are presented as mean ± SEM; n = 6 control and n = 6 NSCLC. (**B**) Relative expression of BCAA metabolic pathway genes in normal pancreas and PDAC tumors from KP mice. Data are presented as mean ± SEM; n = 7 control and n = 5 PDAC. (**C**) Immunoblots of proteins involved in BCAA metabolism in representative normal lung and NSCLC tumors (left) and representative normal pancreas and PDAC tumors (right) from KP mice. (**D**) Quantification of (C). Data are presented as mean ± SEM; n = 6 control and n = 6 NSCLC; n = 4 control and n = 4 PDAC. (**E**) Comparison of BCAA metabolic pathway gene expression in human NSCLC and PDAC tumors relative to their adjacent paired normal tissues. Overall expression of BCAA metabolism genes is significantly decreased in PDAC (P < 0.0001). Two-tailed t test was used for all comparisons between two groups unless otherwise stated. *P < 0.05, **P < 0.01, ***P < 0.001.

^{15}N-amino acid species (Fig. 2B and fig. S5D), this ^{15}N-labeling pattern argues that BCAA-transamination mediated by *Bcat* isoforms is active in NSCLC tumor tissue. Evidence for increased BCAA transamination in NSCLC compared with PDAC cells is also evident in vitro across a range of glutamine concentrations; however, tissue culture does not recapitulate the same phenotypes observed in tumors (fig. S6). Downstream of nonessential amino acid biosynthesis, this nitrogen can also be used to generate nucleotides, primarily if aspartate is synthesized de novo in these tumors. Consistent with this possibility, we found increased incorporation of ^{15}N-label in both aspartate and nucleotides (Fig. 2, C and D, and fig. S5E). In

some contexts, aspartate production is limiting for nucleotide biosynthesis and proliferation (*24*, *25*), indicating that BCAA metabolism may be important for tumor growth.

To test whether gene expression differences might contribute to differential BCAA metabolism, we used quantitative reverse transcription polymerase chain reaction (RT-PCR) to analyze mRNA levels in NSCLC and PDAC tumors compared with their respective normal tissues. Consistent with increased BCAA uptake and KIC generation in NSCLC tumors, these tumors displayed increased expression of the primary BCAA transporter *Slc7a5* (also called the neutral amino acid transporter *Lat1*) and increased levels of *Bcat2* and *Bckdh* (Fig. 3, A, C, and D). In contrast, PDAC exhibited decreased expression of these genes relative to normal pancreas (Fig. 3, B to D). We also observed increased inhibitory phosphorylation of the Bckdh complex in lung tumors (Fig. 3, C and D). *Bcat* expression enables use of BCAAs as a source of nitrogen by lung tumors, and inhibition of Bckdh prevents further catabolism of these amino acids.

The expression changes observed in PDAC are not specific to this model; the related KPC mouse model (*26*), which is initiated by a point mutation in *Trp53*, showed similar changes in gene expression (fig. S7A). Furthermore, these decreases in gene expression do not appear to be a consequence of the relative decrease in cancer cellularity of PDAC tumors (*7*) because sorted pancreatic cancer cells showed similar expression of genes involved in proximal BCAA catabolism relative to whole tumor extracts (fig. S7B). In further agreement with neither lung nor pancreatic cancers showing evidence of downstream BCAA-carbon oxidation, the expression of enzymes from this pathway was not markedly different in either of these cancers (fig. S7, C and D). In contrast, glycolytic gene expression was increased in both tumor types (fig. S7, E and F), which is consistent with known increases in glycolysis in each tumor type (*27*–*29*). Last, to relate these data to tissue of origin, we performed principal component and clustering analyses, which demonstrated segregation of each tumor with the normal tissue from which it arose (figs. S7, G and H).

To ascertain whether similar changes in gene expression were also found in human cancers, we examined expression of BCAA catabolic enzymes in NSCLC and PDAC relative to their respective normal tissues in publicly available data sets (*30*). Consistent with our observations in mice, human NSCLC had increased expression of *SLC7A5*, *BCAT*, and *BCKDH*, whereas expression of BCAA catabolism pathway enzymes was decreased in human PDAC ($P < 0.0001$ for the pathway) (Fig. 3E and tables S1 and S2). The distinct expression patterns for each tumor type were highly correlated across multiple data sets (fig. S7I and tables S3 to S6). The similarity between human NSCLC and the mouse model of NSCLC was observed despite *KRAS* and *TP53* mutations occurring in <50% of human tumors (*8*), and similar expression patterns were also seen in squamous cell lung cancer (fig. S7I and table S6), further supporting the notion that tissue of origin can dictate metabolic phenotype.

The increased contribution of plasma BCAAs to biomass in NSCLC tumors suggests that these tumors may rely on BCAA metabolism for growth. To test this possibility, we used clustered regularly interspaced short palindromic repeats (CRISPR)–Cas9–mediated genome editing to disrupt exon sequences present in both the *Bcat1* (cytosolic) and the *Bcat2* (mitochondrial) isoforms (fig. S8A) in cancer cell lines derived from KP mice with NSCLC (*Bcat* null clones A and B) or PDAC (*Bcat* null) (fig. S8B). Expression analysis and ^{15}N-leucine tracing studies confirmed functional deletion of

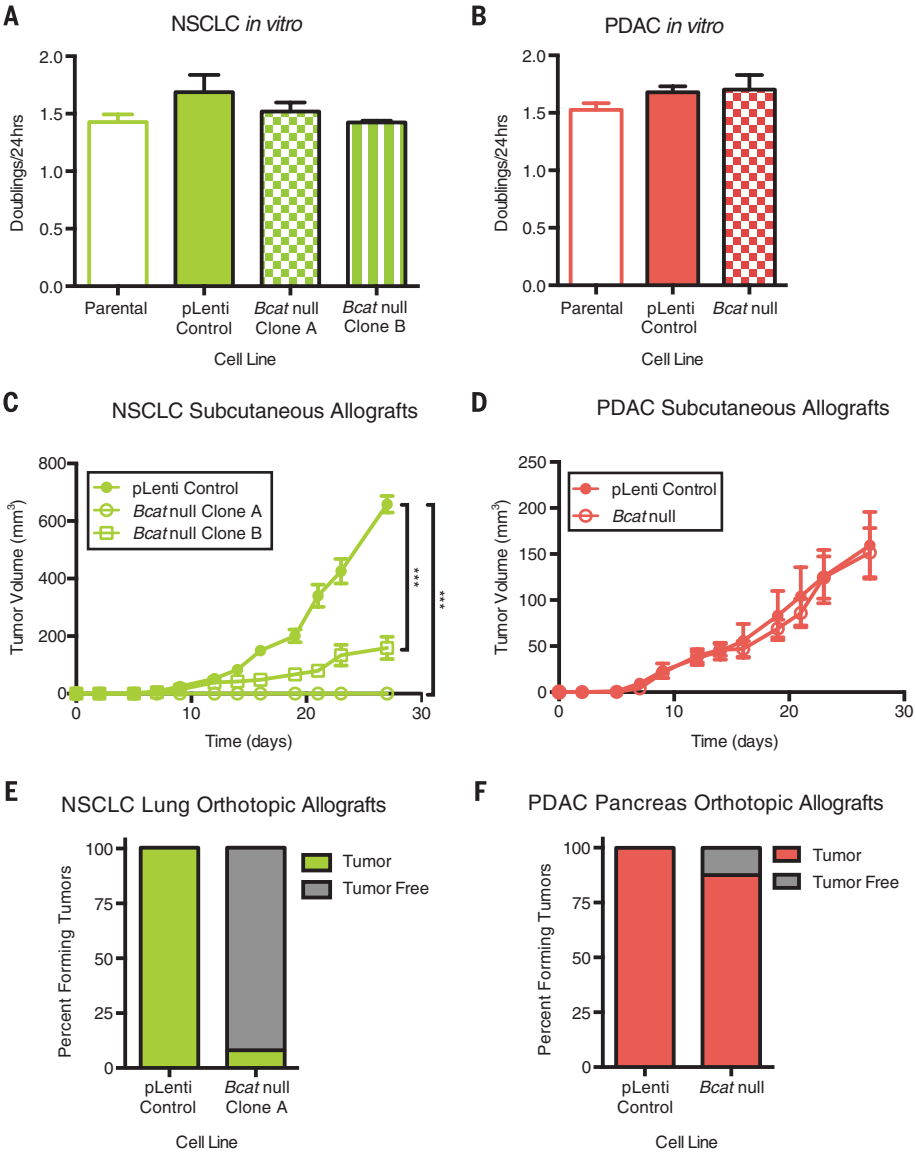


Fig. 4. Bcat activity is required for NSCLC tumor growth. (**A**) Doubling time of parental, control CRISPR-Cas9 vector infected (pLenti), and NSCLC *Bcat* null cell lines in vitro. Data are presented as mean ± SEM; $n = 3$ per group. Representative experiment from ≥2 repeats. (**B**) Doubling time of parental, control CRISPR-Cas9 vector infected, and PDAC *Bcat* null cell lines in vitro. Data are presented as mean ± SEM; $n = 3$ per group. Representative experiment from ≥2 repeats. (**C**) Estimated tumor volume (cubic millimeters) of subcutaneous allograft of control infected and *Bcat* null syngenic NSCLC cell lines into C57BL/6J mice. Data are presented as mean ± SEM; $n = 6$ per group. Two-way repeated measures analysis of variance (ANOVA) was used for comparison between groups. (**D**) Estimated tumor volume (cubic millimeters) of subcutaneous allograft of control infected and *Bcat*-null syngenic PDAC cell lines into C57BL/6J mice. Data are presented as mean ± SEM; $n = 5$ pLenti control and $n = 6$ *Bcat* null. Two-way repeated measures ANOVA was used for comparison between groups. (**E**) Lung orthotopic allograft of control infected and *Bcat*-null syngenic NSCLC cell lines into C57BL/6J mice; $n = 23$ vector control and $n = 13$ *Bcat* null Clone A. (**F**) Pancreatic orthotopic allograft of control infected and *Bcat* null syngenic PDAC cell lines into C57BL/6J mice. $n = 8$ mice per group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Bcat in both the NSCLC and PDAC cancer cells (fig. S8, C to F). Despite loss of both *Bcat* isoforms, these cells proliferate at a rate that is similar to the parental and vector control infected cell lines in vitro (Fig. 4, A and B). When *Bcat*-null NSCLC cells were implanted subcutaneously in vivo, however, the ability of these cells to form tumors was impaired, and one clone failed to produce tumors (Fig. 4C and fig. S8G). In contrast, *Bcat*-null PDAC cells implanted subcutaneously generated tumors (Fig. 4D and fig. S8H). Additionally, orthotopic transplantation of NSCLC *Bcat*-null cells failed to form lung tumors (Fig. 4E), whereas PDAC *Bcat*-null cells formed tumors in the pancreas (Fig. 4F). Unlike subcutaneously implanted PDAC cells in which both *Bcat* isoforms were knocked out, these cells formed smaller tumors in the pancreas than did control cells (fig. S8I). Taken together, these data suggest that although KP lung tumors require Bcat activity for growth, this enzyme activity is dispensable for KP pancreas tumor formation, although PDAC tumor growth may be aided by Bcat activity in some tissue environments.

Proliferating cells need to acquire amino acids, both to make protein and as a source of nitrogen for nucleotide and nonessential amino acid synthesis. Prior work has shown that macropinocytosis plays a role in filling this requirement in mutant *RAS*-driven PDAC tumors and cells (*12*, *14*, *31*). The data presented here argue that this process might be less active in mutant *Ras*–transformed NSCLC tumors that acquire nitrogen in part from free BCAAs. Indeed, we observed less macropinocytosis in cells derived from mouse NSCLC relative to mouse PDAC cells (fig. S9). The decreased reliance of PDAC on free BCAAs, however, does not necessarily imply that uptake of these amino acids would be toxic for this cancer. Overexpressing *Slc7a5* in PDAC cells is sufficient to increase leucine uptake (fig. S10, A and B) but has minimal effects on proliferation in vitro (fig. S10C) or tumor growth in vivo (fig. S10, D and E).

A role for free BCAAs in supplying nitrogen to lung cancers is intriguing in light of recent studies in glioblastoma and NSCLC indicating that glutamine, which is the most abundant plasma amino acid and serves as the major free amino acid substrate for nitrogen and carbon in culture (*32*), contributes less to tumor metabolism in vivo (*33*, *34*). Indeed, glucose-tracing studies in humans and mice demonstrate that glutamine is net synthesized from glucose (*15*, *33*–*37*), and alternative sources of nitrogen are required to support glutamine production. Thus, in these contexts, extraction of nitrogen from BCAAs for de novo amino acid and nucleotide biosynthesis in vivo may explain how lung tumors satisfy their nitrogen requirements. Consistent with this possibility, *BCAT1* expression is known to be important for glioblastoma growth (*38*), suggesting that tumors arising in tissues other than the lung may also use BCAAs as a source of nitrogen. Multiple factors—including local environment, tumor cell of origin, and genetic mutations—can lead to convergent metabolic adaptations in disparate tumor types.

Elevations in plasma BCAA levels are associated with early PDAC and result from increased tissue protein breakdown (*18*). The finding that PDAC tumors have decreased use of circulating BCAAs contributes to this phenotype as well. In contrast, NSCLC tumors actively use BCAAs, leading to plasma BCAA depletions, particularly because the liver does not regulate levels of these amino acids (*23*). Many patients with PDAC and NSCLC tumors develop cachexia with end-stage disease (*39*). Our findings suggest that differential use of amino acids by tumors and the resulting impact on whole-body metabolism might play a role in the initiation and natural history of cachexia. In addition, as personalized medicine plays a larger role in the clinical management of cancer, it will be critical to understand how cell of origin and tissue environment interact with genetic events to influence metabolic dependencies of tumors and select the right treatment approaches for patients.

REFERENCES AND NOTES

1. L. A. Garraway, *J. Clin. Oncol.* **31**, 1806–1814 (2013).
2. N. Vasan, J. L. Boyer, R. S. Herbst, *Clin. Cancer Res.* **20**, 3921–3930 (2014).
3. P. A. Muller, K. H. Vousden, *Cancer Cell* **25**, 304–317 (2014).
4. L. K. Boroughs, R. J. DeBerardinis, *Nat. Cell Biol.* **17**, 351–359 (2015).
5. E. White, *Development* **27**, 2065–2071 (2013).
6. F. Kruiswijk, C. F. Labuschagne, K. H. Vousden, *Nat. Rev. Mol. Cell Biol.* **16**, 393–405 (2015).
7. A. V. Biankin *et al.*, *Nature* **491**, 399–405 (2012).
8. E. A. Collisson *et al.*, *Nature* **511**, 543–550 (2014).
9. P. Vaupel, F. Kallinowski, P. Okunieff, *Cancer Res.* **49**, 6449–6465 (1989).
10. A. Hirayama *et al.*, *Cancer Res.* **69**, 4918–4925 (2009).
11. J. R. Mayers, M. G. Vander Heiden, *Trends Biochem. Sci.* **40**, 130–140 (2015).
12. C. Commisso *et al.*, *Nature* **497**, 633–637 (2013).
13. J. J. Kamphorst *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **110**, 8882–8887 (2013).
14. W. Palm *et al.*, *Cell* **162**, 259–270 (2015).
15. S. M. Davidson *et al.*, *Cell Metab.* **23**, 517–528 (2016).
16. J. Hu *et al.*, *Nat. Biotechnol.* **31**, 522–529 (2013).
17. M. O. Yuneva *et al.*, *Cell Metab.* **15**, 157–170 (2012).
18. J. R. Mayers *et al.*, *Nat. Med.* **20**, 1193–1198 (2014).
19. N. Bardeesy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **103**, 5947–5952 (2006).
20. M. DuPage, A. L. Dooley, T. Jacks, *Nat. Protoc.* **4**, 1064–1072 (2009).
21. A. E. Harper, R. H. Miller, K. P. Block, *Annu. Rev. Nutr.* **4**, 409–454 (1984).
22. C. R. Green *et al.*, *Nat. Chem. Biol.* **12**, 15–21 (2016).
23. J. T. Brosnan, *J. Nutr.* **133** (suppl. 1), 2068S–2072S (2003).
24. K. Birsoy *et al.*, *Cell* **162**, 540–551 (2015).
25. L. B. Sullivan *et al.*, *Cell* **162**, 552–563 (2015).
26. S. R. Hingorani *et al.*, *Cancer Cell* **7**, 469–483 (2005).
27. H. Friess *et al.*, *Gut* **36**, 771–777 (1995).
28. K. Kubota *et al.*, *J. Nucl. Med.* **31**, 1927–1932 (1990).
29. K. B. Nolop *et al.*, *Cancer* **60**, 2682–2689 (1987).
30. T. Barrett *et al.*, *Nucleic Acids Res.* **39** (database), D1005–D1010 (2011).
31. J. J. Kamphorst *et al.*, *Cancer Res.* **75**, 544–553 (2015).
32. R. J. DeBerardinis *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **104**, 19345–19350 (2007).
33. I. Marin-Valencia *et al.*, *Cell Metab.* **15**, 827–837 (2012).
34. K. Sellers *et al.*, *J. Clin. Invest.* **125**, 687–698 (2015).
35. E. A. Maher *et al.*, *NMR Biomed.* **25**, 1234–1244 (2012).
36. S. Tardito *et al.*, *Nat. Cell Biol.* **17**, 1556–1568 (2015).
37. C. T. Hensley *et al.*, *Cell* **164**, 681–694 (2016).
38. M. Tönjes *et al.*, *Nat. Med.* **19**, 901–908 (2013).
39. W. D. Dewys *et al.*, *Am. J. Med.* **69**, 491–497 (1980).

ACKNOWLEDGMENTS

J.R.M. acknowledges support from U.S. NIH National Cancer Institute (NCI) grant F30CA183474 and National Institute of General Medical Sciences grant T32GM007753. A.N.L. is a Robert Black Fellow of the Damon Runyon Cancer Research Foundation, DRG-2241-15. M.G.V.H. acknowledges support from NCI grants P30CA1405141, R01CA168653, and R01CA201276; the Burroughs Wellcome Fund; the Eisen and Chang Families; the Ludwig Center at Massachusetts Institute of Technology; Stand Up To Cancer; and the Lustgarten Foundation. M.G.V.H. is a paid scientific adviser for Agios Pharmaceuticals and for Aeglea Biotherapeutics, biotechnology companies that are developing drugs targeting cellular metabolism to treat cancer and other diseases.

SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6304/1161/suppl/DC1
Materials and Methods
Figs. S1 to S10
Tables S1 to S8
References (*40*–*49*)

21 February 2016; accepted 29 July 2016
10.1126/science.aaf5171

Tissue of origin dictates branched-chain amino acid metabolism in mutant *Kras*-driven cancers

Jared R. Mayers, Margaret E. Torrence, Laura V. Danai, Thales Papagiannakopoulos, Shawn M. Davidson, Matthew R. Bauer, Allison N. Lau, Brian W. Ji, Purushottam D. Dixit, Aaron M. Hosios, Alexander Muir, Christopher R. Chin, Elizaveta Freinkman, Tyler Jacks, Brian M. Wolpin, Dennis Vitkup and Matthew G. Vander Heiden (September 8, 2016)

Science **353** (6304), 1161-1165. [doi: 10.1126/science.aaf5171]

Editor's Summary

Science (print ISSN 0036-8075; online ISSN 1095-9203) is published weekly, except the last week in December, by the American Association for the Advancement of Science, 1200 New York Avenue NW, Washington, DC 20005.

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

Research Article

Cancer Epidemiology, Biomarkers & Prevention

Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics

Mary K. Townsend[1], Ying Bao[1], Elizabeth M. Poole[1], Kimberly A. Bertrand[2], Peter Kraft[3], Brian M. Wolpin[4,5], Clary B. Clish[6], and Shelley S. Tworoger[1,3]

Abstract

Background: Many epidemiologic studies are using metabolomics to discover markers of carcinogenesis. However, limited data are available on the influence of pre-analytic blood collection factors on metabolite measurement.

Methods: We quantified 166 metabolites in archived plasma from 423 Health Professionals Follow-up Study and Nurses' Health Study participants using liquid chromatography–tandem mass spectrometry (LC-MS). We compared multivariable-adjusted geometric mean metabolite LC-MS peak areas across fasting time, season of blood collection, and time of day of blood collection categories.

Results: The majority of metabolites (160 of 166 metabolites) had geometric mean peak areas that were within 15% comparing samples donated after fasting 9 to 12 versus ≥13 hours; greater differences were observed in samples donated after fasting ≤4 hours. Metabolite peak areas generally were similar across season of blood collection, although levels of certain metabolites (e.g., bile acids and purines/pyrimidines) tended to be different in the summer versus winter months. After adjusting for fasting status, geometric mean peak areas for bile acids and vitamins, but not other metabolites, differed by time of day of blood collection.

Conclusion: Fasting, season of blood collection, and time of day of blood collection were not important sources of variability in measurements of most metabolites in our study. However, considering blood collection variables in the design or analysis of studies may be important for certain specific metabolites, particularly bile acids, purines/pyrimidines, and vitamins.

Impact: These results may be useful for investigators formulating analysis plans for epidemiologic metabolomics studies, including determining which metabolites to *a priori* exclude from analyses. *Cancer Epidemiol Biomarkers Prev; 25(5); 823–9. ©2016 AACR.*

Introduction

Metabolomic profiling of archived biospecimens from participants enrolled in epidemiologic studies has the potential to facilitate discovery of early biologic markers of carcinogenesis as well as markers of cancer prognosis (1). However, conducting a large-scale biospecimen collection can be challenging, particularly when participants are spread across a wide geographic area, and standardizing certain aspects of the blood collection protocol across participants may be particularly difficult. For example, in an uncontrolled setting, some participants may not be able to follow strict protocols with regard to fasting prior to blood draw or time of day of blood collection. Additionally, blood collections in large populations may span several months, resulting in different seasons of blood collection across participants, and thus seasonal differences in exposures (e.g., diet or light exposure) that may affect biomarker levels.

Few studies have evaluated whether levels of plasma metabolites measured by metabolomics profiling platforms vary by pre-analytic blood sample collection factors, such as fasting time, time of day of blood collection, or season of blood collection (2–9). Such information is necessary to understand the importance of controlling for these variables in the design of metabolomics studies (e.g., matching cases and controls on season of blood draw or restricting the study population to those fasting at least 8 hours) or during data analysis (e.g., excluding certain metabolites likely to be inaccurately measured based on pre-analytic variables). Failure to adequately consider these sources of measurement error could lead to decreased power of statistical tests and biased association measures (10). We evaluated differences in plasma metabolite peak areas across participants in two large, prospective cohort studies with different fasting times at blood draw, times of day of blood draw, and seasons of blood draw. Our analyses considered data on 166 metabolites, measured by a liquid chromatography–tandem mass spectrometry (LC-MS) metabolomics platform, which performed well in our previous pilot testing (11).

[1]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [2]Slone Epidemiology Center, Boston University, Boston, Massachusetts. [3]Department of Epidemiology, Harvard T.H. Chan School of Public Health, Boston, Massachusetts. [4]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. [5]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts. [6]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, Massachusetts.

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

M.K. Townsend and Y. Bao contributed equally to this article and share first authorship.

C.B. Clish and S.S. Tworoger contributed equally to this article and share senior authorship.

Corresponding Author: Mary K. Townsend, Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115. Phone: 617-525-2764; Fax: 617-525-2008; E-mail: nhmkt@channing.harvard.edu

doi: 10.1158/1055-9965.EPI-15-1206

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

Materials and Methods

Study population

The Nurses' Health Study (NHS) was established in 1976 when 121,700 female nurses ages 30 to 55 years completed a mailed questionnaire. The Health Professionals Follow-up Study (HPFS) was initiated in 1986 when 51,529 men ages 40 to 75 years and working in health professions completed a mailed questionnaire. Both cohorts enrolled only adults living in the United States and have followed participants since enrollment via mailed biennial questionnaires. Blood samples were collected in heparin tubes from 32,826 women in NHS from 1989 to 1990 and in EDTA tubes from 18,225 men in HPFS from 1993 to 1995. Participants arranged to have their blood collected and shipped the samples via overnight mail with an icepack to a central laboratory. On arrival, the samples were processed and aliquots of plasma, white blood cells, and red blood cells were stored in liquid nitrogen freezers; >95% of samples arrived within 24 hours of collection (12, 13). Participants provided information on the date of blood collection, time at which blood was drawn, and number of hours since last eating before blood draw on a questionnaire returned with the samples.

Our analyses included 423 participants (252 from NHS and 171 from HPFS) selected as controls for a previous study of plasma metabolomics and pancreatic cancer (14). All participants provided implied consent by returning the questionnaires and blood samples. The study was approved by the Human Research Committee at Brigham and Women's Hospital (Boston, MA).

Metabolite profiling

Profiles of 231 endogenous polar metabolites and lipids were obtained using LC-MS at the Broad Institute of the Massachusetts Institute of Technology and Harvard University (Cambridge, MA). A detailed description of the metabolite profiling methods has been previously published (14). For the current analysis, we excluded 35 metabolites that were not reasonably reproducible in samples processed up to 24 hours after blood draw (due to the design of our collections) in previous pilot tests (11, 14). Reasonable reproducibility was defined as an intraclass correlation (ICC) $\geq$0.75 comparing samples processed immediately versus 24 hours later (15). In addition, we excluded 15 metabolites with inter-assay coefficients of variation >25%, as measured in blinded quality control samples, and 15 metabolites with undetectable levels in >10% of participants. Thus, 166 metabolites were retained for these analyses.

Statistical analysis

Metabolite levels were reported as measured LC-MS peak areas, which are proportional to metabolite concentration. We grouped metabolites into 9 categories: amines ($n = 5$); amino acid derivatives ($n = 13$); amino acids ($n = 28$); bile acids ($n = 5$); lipids and lipid metabolites ($n = 88$); organic acids ($n = 11$); purines, pyrimidines, and derivatives ($n = 3$); vitamins ($n = 3$); and other ($n = 10$; see Supplementary Table S1 for the list of metabolites within each category). To quantify the strength of the relationship between metabolites in each category, after adjusting for potential differences between participants that might affect metabolite levels, we calculated Spearman partial correlation coefficients. The covariates included age at blood draw (years, continuous), race (white, non-white), body mass index at blood draw (continuous, kg/m^2), physical activity (metabolic equivalent hours/week, continuous), sex/menopausal status/hormone use (male, female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use), and total caloric intake (kcal, continuous). Data on covariates were obtained from the cohort questionnaire closest to the date of blood collection.

For the analyses of metabolite levels according to blood sample collection factors, each metabolite peak area was log-transformed to improve the normality of its distribution and included as the dependent variable in a robust variance linear regression model (PROC MIXED, version 9.3; SAS Institute). The primary independent variable in the model was the pre-analytic blood collection variable of interest: fasting time [$\leq$4 (referent), 5–8, 9–12, $\geq$13 hours], season of blood draw [February–April (referent), May–July, August–October, November–January], or time of day of blood draw [6–8 am (referent), 9–10 am, 11 am–12 pm, 1–2 pm, 3 pm–12 am]. We calculated the mean log-transformed metabolite peak area in each category of the blood collection variable after adjusting for the covariates (i.e., marginal means). We then calculated multivariable-adjusted geometric mean peak areas by taking the anti-log of each marginal mean. We calculated the percentage difference in geometric mean metabolite peak area between each category of the blood collection variable and the referent category (e.g., fasting 9–12, 5–8, or $\leq$4 hours vs. $\geq$13 hours) by subtracting the referent geometric mean peak area from the geometric mean peak area of the category of interest and dividing by the referent geometric mean peak area.

Within each metabolite category, we calculated the median as well as the 10th and 90th percentiles of the percentage differences in the geometric mean metabolite peak area for each blood collection variable category (except the referent group). We also calculated the percentage (95% confidence interval) of metabolites in each metabolite category whose geometric mean peak areas were $\pm$15% of the referent category geometric mean and considered alternate cutoff points (10%, 20%) in sensitivity analyses.

Because fasting time and time of day of blood draw are highly correlated, analyses of time of day of blood draw were further adjusted for fasting time ($\leq$4, 5–8, 9–12, $\geq$13 hours). Also, to further explore the impact of fasting time on associations between time of day of blood draw and metabolite concentration, we conducted separate analyses among individuals fasting $\leq$8 hours and those fasting >8 hours. Due to the smaller sample size, time of day of blood draw was evaluated in three categories in these analyses (6–8 am, 9 am–12 pm, 1 pm–12 am).

Results

The mean age at blood draw was 65 years among men in HPFS and 60 years among women in NHS (Table 1). Among both men and women, a large majority were white (94% in HPFS; 99% in NHS). The most common season of blood collection was late summer/early autumn among men (46% in August–October) and late winter/early spring among women (36% in February–April). The majority of participants in both cohorts had fasted for more than 8 hours at blood collection and had their blood drawn in the morning.

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

Table 1. Age-standardized characteristics of the study population[a]

Characteristic	HPFS ($n = 171$)	NHS ($n = 252$)
Age at blood draw, y, mean (SD)	65.2 (7.8)	60.2 (6.2)
White, %	94.4	99.4
Height, inches, mean (SD)	70.1 (2.9)	64.4 (2.4)
Body mass index, kg/m^2, mean (SD)	26.0 (3.2)	25.9 (5.0)
Physical activity, MET-hr/wk, mean (SD)	38.0 (35.1)	17.7 (20.3)
Season of blood draw, %		
Feb–Apr	10.0	36.2
May–Jul	27.7	31.4
Aug–Oct	45.9	15.1
Nov–Jan	16.4	17.3
Fasting time at blood draw, %		
≤4 h	31.9	18.5
5–8 h	11.6	5.0
9–12 h	36.8	45.7
≥13 h	19.7	30.8
Time of day of blood draw, %		
6–8 am	29.9	37.7
9–10 am	37.0	45.7
11 am-noon	9.9	7.6
1–2 pm	11.6	5.2
3 pm-midnight	11.6	3.8

Abbreviations: MET, metabolic equivalent; SD, standard deviation.
[a]All values (except age at blood draw) are standardized to the age distribution of the study population.

The distribution of interassay coefficients of variation for metabolites in each category is shown in Table 2. Spearman partial correlation coefficients among amines, amino acid derivatives, and purines and pyrimidines were weak to moderate (Supplementary Table S2). Correlations among amino acids, bile acids, lipids, and organic acids ranged from nearly 0 to >0.8.

Metabolite peak areas were similar across categories of fasting time for the majority of metabolites, particularly among people who had last eaten >4 hours prior to blood draw (Table 3). Specifically, geometric mean metabolite peak areas measured in samples collected after 9 to12 or 5 to 8 hours fasting were within 15% of geometric mean metabolite peak areas in samples collected after fasting at least 13 hours for 91% to 100% of amino acid derivatives, amino acids, lipids and lipid metabolites, organic acids, and purines and pyrimidines. Metabolite peak areas were less similar when comparing samples from participants fasting ≤4 versus ≥13 hours. In particular, geometric mean peak areas comparing participants fasting ≤4 versus ≥13 hours were within 15% for only 1 of 5 bile acids and none of the 3 vitamins. Supplementary Table S1 shows the percentage differences in geometric mean peak areas by fasting time for each metabolite. Results for the 10% and 20% cutoffs were similar to the 15% cutoff (Supplementary Table S3).

Geometric mean peak areas of most metabolites were similar across seasons of blood collection (Table 4 and Supplementary Table S4). Among participants whose blood was collected in May–July, August–October, or November–January, geometric mean peak areas for at least 80% of amines, amino acid derivatives, amino acids, and lipids and lipid metabolites were within 15% of those among participants whose blood was collected between February and April. However, levels of certain types of metabolites appeared to vary by season. For example, geometric mean metabolite peak areas in samples collected during peak sun months (August–October) were within 15% of those collected during low sun months (February–April) for just 67% of purines/pyrimidines, 55% of organic acids, and none of the bile acids. When using the more conservative criterion of percentage difference ≤10%, these figures were 33%, 55%, and 0%, respectively (Supplementary Table S5).

We investigated percentage differences in geometric mean metabolite peak areas by time of day of blood collection adjusting for fasting time (Table 5 and Supplementary Tables S6 and S7). Geometric mean levels of amino acid derivatives, amino acids, lipids and lipid metabolites, organic acids, and purines and pyrimidines generally were similar across individuals whose blood was collected at different times during the day. However, levels of certain metabolites, such as bile acids and vitamins, appeared to be more variable during the day. To further examine these patterns, we conducted additional analyses separately among participants fasting ≤8 hours and participants fasting >8 hours (Supplementary Table S8). Differences in geometric mean metabolite peak areas for bile acids by time of day of blood draw were evident among both participants fasting >8 and ≤8 hours. Among participants fasting >8 hours, bile acid levels tended to be lower in those whose time of blood draw was 9 am–12 pm or 1 pm–12 am versus 6–8 am (percentage differences, 10th to 90th percentiles = −25%, −32% to −14%, and −15%, −52% to −4%, respectively). Differences in vitamin peak areas by time of day of blood draw were mainly apparent among participants fasting <8 hours; vitamin levels tended to be higher among those who blood was drawn between 9 am and 12 am versus 6–8 am.

Discussion

Overall, our results suggest that, after excluding metabolites that are not stable with delays in processing up to 24 hours, fasting time, time of day of blood draw, and season of blood draw are not important sources of variability for the majority of metabolites measured by our metabolomics profiling platform, particularly amines, amino acids, amino acid derivatives, and lipids and lipid metabolites. However, our results also suggest that careful attention to pre-analytic variables related to blood collection may be necessary in analyses focused on certain types of metabolites. Specifically, we observed variability in levels of organic acids, purines and pyrimidines, bile acids, and vitamins by fasting

Table 2. Distribution of inter-assay coefficients of variation for metabolites in each category[a]

Metabolite category	Metabolites, *n*	Median (minimum – maximum) interassay coefficient of variation (%)
Amines	5	8.9 (8.2–22.5)
Amino acid derivatives	13	13.0 (9.6–23.9)
Amino acids	28	10.4 (6.9–18.0)
Bile acids	5	17.6 (12.1–20.5)
Lipids and lipid metabolites	88	9.6 (4.0–25.0)
Organic acids	11	13.3 (5.2–24.8)
Purines, pyrimidines, and derivatives	3	9.0 (6.8–20.5)
Vitamins	3	14.8 (11.5–16.1)
Other[b]	10	10.5 (6.6–16.3)

[a]Metabolites with an interassay coefficient of variation >25% were excluded from analysis.
[b]Other metabolites include allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine, salicylurate, sorbitol, and uracil.

Table 3. Percentage difference in geometric mean metabolite LC-MS peak areas, by fasting time[a]

			Fasting 9–12 vs. ≥13 h		Fasting 5–8 vs. ≥13 h		Fasting ≤4 vs. ≥13 h	
Metabolite category	*N*	Fasting ≥13 h	Percentage difference, median (10th–90th percentiles)	Percentage of metabolites within 15% of referent	Percentage difference, median (10th–90th percentiles)	Percentage of metabolites within 15% of referent	Percentage difference, median (10th–90th percentiles)	Percentage of metabolites within 15% of referent
Amines	5	Referent	−2.7 (−10.6 to 2.4)	100%	−4.6 (−21.0 to 6.5)	80%	−2.9 (−16.3 to 6.7)	80%
Amino acid derivatives	13	Referent	1.5 (−5.5 to 8.2)	100%	−2.7 (−11.4 to 3.7)	92%	4.8 (−3.8 to 24.6)	77%
Amino acids	28	Referent	2.0 (−3.8 to 5.3)	96%	−2.4 (−9.4 to 10.8)	96%	2.8 (−5.5 to 20.7)	86%
Bile acids	5	Referent	0.4 (−8.4 to 17.4)	80%	−11.3 (−29.1 to 18.7)	60%	53.1 (3.0 to 129.9)	20%
Lipids and lipid metabolites	88	Referent	0.2 (−4.6 to 8.6)	98%	0.5 (−3.8 to 10.7)	92%	0.9 (−4.5 to 26.4)	83%
Organic acids	11	Referent	−5.1 (−13.7 to 3.5)	91%	0.6 (−4.3 to 10.2)	91%	−0.6 (−18.1 to 10.7)	73%
Purines, pyrimidines, and derivatives	3	Referent	−1.2 (−2.9 to 1.8)	100%	−3.9 (−7.4 to −1.7)	100%	−2.6 (−26.6 to 0.7)	67%
Vitamins	3	Referent	7.6 (0.8 to 10.4)	100%	9.6 (9.2 to 16.0)	67%	58.6 (24.1 to 77.3)	0%
Other[b]	10	Referent	−0.4 (−16.4 to 10.7)	90%	−2.8 (−24.0 to 18.6)	50%	12.4 (−24.2 to 84.5)	30%

NOTE: *N*, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hr/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), and total caloric intake (kcal, continuous).

[b]Other metabolites include allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine, salicylurate, sorbitol, and uracil.

status, especially when comparing participants who ate relatively recently (fasting time ≤4 hours) to those whose last meal was more distant (fasting time ≥13 hours). Regarding differences by season of blood draw, we observed a pattern in which measurements of bile acids, organic acids, and purines and pyrimidines were most variable between months with peak sun (May–October) versus low sun (February–April) in the Northern Hemisphere, where the large majority of participants in our study reside. Finally, after adjusting for fasting time, having blood drawn in the late morning, afternoon, or evening compared with the early morning (6–8 am) was associated with variability in measured peak areas for bile acids and vitamins, although variation in vitamin levels by time of day of blood draw was restricted mainly to individuals fasting <8 hours.

Few previous studies have investigated differences in metabolite profiles by pre-analytic variables related to blood collection. Brauer and colleagues conducted amino acid metabolite profiling using electrospray tandem–mass spectrometry in whole blood collected from 10 adults after fasting >5 hours as well as 3 and 5 hours after eating a standardized meal (5). They observed changes in amino acid levels 3 and 5 hours postprandial, suggesting that a fasting period longer than 5 hours should be considered for measurement of amino acids. Similarly, in our analysis, fasting time between 5 and 12 hours compared with ≥13 hours appeared to have minimal influence on levels of amino acids. We observed more differences in amino acid levels when comparing samples from participants fasting ≤4 hours versus ≥13 hours, although percentage differences in geometric mean peak areas remained within 15% for the large majority (86%) of amino acids. In our study, bile acids and vitamins were the most variable types of metabolites across fasting time categories and tended to be higher in participants fasting ≤4 hours versus ≥13 hours. This pattern likely reflects the intake of nutrients and reabsorption of bile acids from the intestine following ingestion of food (16). In general, our results regarding variability in metabolite levels by fasting status are congruent with the conclusion of Sampson and colleagues that fasting accounts for little of the variability in metabolites and, therefore, metabolomics can be a useful tool even in studies without strict guidelines on fasting before blood draws (4).

Few data have been published on potential seasonal differences in metabolite profiles. The differences we observed in levels of bile acids, organic acids, and purines and pyrimidines across individuals with different seasons of blood draw might reflect seasonal variation in diet or other exposures, such as sunlight. Interestingly, the pattern we observed of higher urate levels among participants who donated blood in the summer versus winter months has been previously reported, although the mechanism underlying this pattern is not clear (17). Overall, season did not appear to be an important source of variability for most types of metabolites in our study.

Several studies have reported on diurnal variation in biomarker concentrations. Ang and colleagues observed significant 24-hour variation in six amino acids (leucine, lysine, methionine, phenylalanine, proline, and tyrosine) in a laboratory study of 8 men who consumed a nutritional drink every hour while awake (3). Similarly, in our analysis of individuals who fasted ≤8 hours before blood draw, the percentage difference in geometric mean metabolite peak area comparing individuals who donated blood between 1 pm and 12 am with those who donated blood between

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

Table 4. Percentage difference in geometric mean metabolite LC-MS peak areas, by season of blood draw[a]

			May–Jul vs. Feb–Apr		Aug–Oct vs. Feb–Apr		Nov–Jan vs. Feb–Apr	
Metabolite category	***N***	**Feb–Apr**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**
Amines	5	Referent	−1.2 (−13.4 to 9.5)	100%	3.7 (−13.9 to 12.1)	100%	−2.0 (−15.8 to 3.0)	80%
Amino acid derivatives	13	Referent	2.5 (−2.4 to 6.0)	100%	6.2 (1.6 to 14.0)	100%	−4.4 (−9.9 to 3.1)	100%
Amino acids	28	Referent	1.7 (−2.7 to 19.0)	86%	5.3 (−0.8 to 23.9)	82%	−3.2 (−6.4 to 2.6)	100%
Bile acids	5	Referent	13.7 (0.2 to 27.2)	80%	45.7 (21.2 to 53.7)	0%	2.5 (−6.6 to 30.2)	80%
Lipids and lipid metabolites	88	Referent	−1.4 (−4.7 to 3.4)	100%	−2.8 (−9.9 to 3.6)	98%	−1.6 (−7.6 to 2.1)	100%
Organic acids	11	Referent	5.4 (−0.6 to 37.3)	64%	6.2 (0.1 to 35.0)	55%	−0.1 (−1.9 to 9.5)	91%
Purines, pyrimidines, and derivatives	3	Referent	5.9 (0.5 to 8.4)	100%	10.9 (0.5 to 20.4)	67%	3.1 (−0.4 to 8.7)	100%
Vitamins	3	Referent	15.4 (3.9 to 16.9)	33%	7.6 (−5.0 to 12.3)	100%	2.7 (−1.1 to 27.3)	67%
Other[b]	10	Referent	4.3 (−26.8 to 21.3)	50%	4.9 (−4.4 to 19.6)	90%	−2.0 (−17.2 to 4.0)	90%

NOTE: *N*, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hr/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), and total caloric intake (kcal, continuous).

[b]Other metabolites include allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine, salicylurate, sorbitol, and uracil.

Table 5. Percentage difference in geometric mean metabolite LC-MS peak areas, by time of day of blood draw[a]

			9–10 am vs. 6–8 am		11 am–noon vs. 6–8 am		1–2 pm vs. 6–8 am		3 pm–midnight vs. 6–8 am	
Metabolite category	***N***	**6–8 am**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**	**Percentage difference, median (10th–90th percentiles)**	**Percentage of metabolites within 15% of referent**
Amines	5	Ref	2.5 (1.8 to 8.5)	100%	2.2 (−10.1 to 19.0)	80%	0.4 (−30.4 to 28.1)	60%	0.6 (−6.0 to 11.3)	100%
Amino acid derivatives	13	Ref	5.4 (0.3 to 8.4)	100%	5.9 (−4.4 to 10.5)	100%	0.8 (−9.7 to 6.3)	92%	2.7 (−7.5 to 17.8)	85%
Amino acids	28	Ref	−3.5 (−6.3 to 2.0)	100%	−8.2 (−15.4 to −0.1)	89%	−7.4 (−14.4 to 6.7)	89%	−6.3 (−15.5 to 7.1)	82%
Bile acids	5	Ref	−21.7 (−29.6 to −9.8)	20%	−28 (−39.2 to −19.4)	0%	3.1 (−41.5 to 9.1)	80%	−10.7 (−39.2 to −1.0)	60%
Lipids and lipid metabolites	88	Ref	0.4 (−2.9 to 2.6)	100%	2.7 (−5.1 to 12.5)	94%	0.7 (−7.5 to 10.4)	93%	−1.6 (−10.5 to 4.8)	92%
Organic acids	11	Ref	1.4 (−0.3 to 9.1)	100%	−0.4 (−10.3 to 10.8)	91%	1.3 (−2.9 to 30.2)	64%	5.2 (0.9 to 27.0)	83%
Purines and pyrimidines	3	Ref	−0.9 (−3.7 to 22.4)	67%	7.3 (5.1 to 8.3)	100%	−1.7 (−3.5 to −1.4)	100%	−4.7 (−9.7 to 2.2)	100%
Vitamins	3	Ref	17.6 (12.4 to 24.8)	33%	−13.0 (−16.9 to 3.0)	67%	17.0 (14.3 to 36)	33%	13.7 (−8.9 to 47.7)	67%
Other[b]	10	Ref	−2.4 (−17.8 to 23.7)	80%	−2.0 (−23.4 to 61.7)	60%	2.2 (−22.3 to 56.5)	60%	9.1 (−17.1 to 187.2)	60%

NOTE: *N*, number of metabolites.

[a]Geometric means were adjusted for age at blood draw (years, continuous), race (white, non-white), body mass index (continuous, kg/m^2), physical activity (metabolic equivalent-hr/wk, continuous), sex/menopausal status (female/premenopausal, female/postmenopausal and no hormone therapy use, female/postmenopausal and hormone therapy use, male), total caloric intake (kcal, continuous), and fasting time ($\leq$4, 5–8, 9–12, $\geq$13 hours).

[b]Other metabolites include allantoin, cotinine, creatine, creatinine, hippurate, hydroxyphenylacetate, phenylethylamine, salicylurate, sorbitol, and uracil.

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

6 am and 8 am was >15% for four of the six amino acids highlighted by Ang and colleagues (data not shown). However, we did not observe large differences in the six amino acids by time of day of blood draw among participants who had fasted more than 8 hours at blood draw (all percentage differences <10%, data not shown). The differences we observed in bile acids by time of day of blood draw among participants fasting ≤8 hours are consistent with known changes in concentrations of circulating bile acids following meal ingestion. In addition, serum levels of bile acids have been shown to decrease during the day among fasting individuals (18), consistent with our results among individuals fasting >8 hours. The differences we observed in levels of vitamins by time of day of blood draw, only among participants fasting <8 hours, also likely reflect food ingestion. However, it should be noted that the three vitamins included in our analysis were B-complex vitamins, which are water-soluble and not stored in the body. Thus, our results likely do not reflect variability in levels of fat-soluble vitamins during the day.

Several limitations of our study should be considered. First, we examined differences in metabolite levels across participants with different blood collection characteristics, rather than differences within individuals observed under different conditions. However, we adjusted the mean metabolite peak areas for a variety of individual-level characteristics (e.g., sex, age, and body mass index) that might influence metabolite levels and our findings are generally consistent with those observed in the few clinic-based studies. Still, it is possible that some of our novel findings, particularly those related to season of blood draw, are due to chance and should be confirmed in future studies. In addition, we excluded 35 metabolites from analysis that were not stable with delays in processing up to 24 hours, as demonstrated in our previous pilot testing (11), in order to focus on metabolites likely to be informative in our study population. Thus, some of the metabolite categories in our analysis contained small numbers of metabolites (e.g., purines and pyrimidines, vitamins) and certain categories were excluded entirely (e.g., carbohydrates); this limits our ability to make conclusions about the influence of blood collection characteristics on certain metabolite types. Finally, several factors may limit the generalizability of our results. Our primary analyses summarized results within metabolite categories. Correlations between metabolites within categories will influence the amount of variability in percentage differences observed (Supplementary Table S2). Therefore, our results may not be generalizable to other metabolomics platforms in which correlations between metabolites differ from those in our study or platforms that measured different sets of metabolites. Also, our results may not apply to study populations that differ significantly from the generally healthy, middle- and older-aged adults in our study.

In conclusion, the results of our analysis suggest that fasting time, season of blood draw, and time of day of blood draw are not important sources of variability for several major classes of metabolites measured by an LC-MS metabolomics platform, including amines, amino acid derivatives, amino acids, and lipids and lipid metabolites. Thus, epidemiologic studies with archived blood specimens may be useful settings for future metabolomics research, even if strict blood collection protocols were not instituted or data on blood collection characteristics are not available. However, our results also suggest that sample collection factors may be important to consider if certain metabolite classes are of particular scientific interest. Investigation of water-soluble vitamins should be restricted to individuals fasting at least 8 hours. In addition, evaluating a potential influence of season of blood draw on vitamin levels may be prudent (e.g., in sensitivity analyses). Due to variability by season and time of day of blood draw, analyses of bile acids may be difficult in epidemiologic studies. If bile acids are of particular interest, analyses should be limited to participants fasting at least 8 hours at blood draw. Overall, our results may be useful for epidemiologic studies to *a priori* exclude metabolites subject to substantial measurement error from analyses, and thus reduce penalties for multiple testing, and when considering variables related to blood collection to include in analysis plans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of Harvard Catalyst, Harvard University and its affiliated academic healthcare centers, or the NIH.

Authors' Contributions

Conception and design: Y. Bao, K.A. Bertrand, P. Kraft, B.M. Wolpin, S.S. Tworoger

Development of methodology: Y. Bao, K.A. Bertrand, B.M. Wolpin, C.B. Clish

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.B. Clish, S.S. Tworoger

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.K. Townsend, Y. Bao, E.M. Poole, K.A. Bertrand, P. Kraft, B.M. Wolpin, S.S. Tworoger

Writing, review, and/or revision of the manuscript: M.K. Townsend, Y. Bao, E.M. Poole, K.A. Bertrand, P. Kraft, B.M. Wolpin, C.B. Clish, S.S. Tworoger

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Bao, E.M. Poole, S.S. Tworoger

Study supervision: S.S. Tworoger

Acknowledgments

The authors acknowledge A. Deik and K. Bullock of the Broad Institute for assistance with LC-MS sample analyses.

Grant Support

The Nurses' Health Study and the Health Professionals Follow-up Study are supported by grants UM1 CA186107, P01 CA87969, R01 CA49449, and UM1 CA167552 from the NCI of the NIH. Y. Bao was supported by grants U54 CA155626 from the NCI and P30 DK046200 from the National Institute of Diabetes and Digestive and Kidney Diseases of the NIH, and a KL2/Catalyst Medical Research Investigator Training award (an appointed KL2 award) from Harvard Catalyst | The Harvard Clinical and Translational Science Center (National Center for Research Resources and the National Center for Advancing Translational Sciences, NIH Award KL2 TR001100). M.K. Townsend was supported by grant K01 HL125698 from the National Heart, Lung, and Blood Institute of the NIH. B.M. Wolpin was supported by grants K07 CA140790 from the NCI of the NIH, the American Society of Clinical Oncology Conquer Cancer Foundation, the Howard Hughes Medical Institute, Promises for Purple, and the Lustgarten Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 2, 2015; revised February 18, 2016; accepted February 18, 2016; published OnlineFirst March 3, 2016.

References

1. Patel S, Ahmed S. Emerging field of metabolomics: big promise for cancer biomarker identification and drug discovery. J Pharm Biomed Anal 2015;107:63–74.
2. Kim K, Mall C, Taylor SL, Hitchcock S, Zhang C, Wettersten HI, et al. Mealtime, temporal, and daily variability of the human urinary and plasma metabolomes in a tightly controlled environment. PLoS One 2014;9: e86223.
3. Ang JE, Revell V, Mann A, Mantele S, Otway DT, Johnston JD, et al. Identification of human plasma metabolites exhibiting time-of-day variation using an untargeted liquid chromatography-mass spectrometry metabolomic approach. Chronobiol Int 2012;29:868–81.
4. Sampson JN, Boca SM, Shu XO, Stolzenberg-Solomon RZ, Matthews CE, Hsing AW, et al. Metabolomics in epidemiology: sources of variability in metabolite measurements and implications. Cancer Epidemiol Biomarkers Prev 2013;22:631–40.
5. Brauer R, Leichtle AB, Fiedler GM, Thiery J, Ceglarek U. Preanalytical standardization of amino acid and acylcarnitine metabolite profiling in human blood using tandem mass spectrometry. Metabolomics 2011;7:344–52.
6. Yin P, Lehmann R, Xu G. Effects of pre-analytical processes on blood samples used in metabolomics studies. Anal Bioanal Chem 2015; 407:4879–92.
7. Breier M, Wahl S, Prehn C, Fugmann M, Ferrari U, Weise M, et al. Targeted metabolomics identifies reliable and stable metabolites in human serum and plasma samples. PLoS One 2014;9:e89728.
8. Kamlage B, Maldonado SG, Bethan B, Peter E, Schmitz O, Liebenberg V, et al. Quality markers addressing preanalytical variations of blood and plasma processing identified by broad and targeted metabolite profiling. Clin Chem 2014;60:399–412.
9. Yin P, Peter A, Franken H, Zhao X, Neukamm SS, Rosenbaum L, et al. Preanalytical aspects and sample quality assessment in metabolomics studies of human blood. Clin Chem 2013;59:833–45.
10. White E. Measurement error in biomarkers: sources, assessment, and impact on studies. In: Rothman N, Hainaut P, Schulte P, Smith M, Boffetta P, Perera F, editors. Molecular epidemiology: principles and practices . Lyon: International Agency for Research on Cancer; 2011. p.143–61.
11. Townsend MK, Clish CB, Kraft P, Wu C, Souza AL, Deik AA, et al. Reproducibility of metabolomic profiles among men and women in 2 large cohort studies. Clin Chem 2013;59:1657–67.
12. Hankinson SE, Willett WC, Manson JE, Colditz GA, Hunter DJ, Spiegelman D, et al. Plasma sex steroid hormone levels and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 1998;90:1292–9.
13. Chu NF, Spiegelman D, Yu J, Rifai N, Hotamisligil GS, Rimm EB. Plasma leptin concentrations and four-year weight gain among US men. Int J Obes Relat Metab Disord 2001;25:346–53.
14. Mayers JR, Wu C, Clish CB, Kraft P, Torrence ME, Fiske BP, et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. Nat Med 2014;20:1193–8.
15. Rosner B. Fundamentals of biostatistics . 7th ed. Boston: Cengage Learning; 2011.
16. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bile-acid signalling for metabolic diseases. Nat Rev Drug Discov 2008;7:678–93.
17. Murciano Revert J, Martinez-Lahuerta JJ, Aleixandre Porcar L. [Seasonal change in blood concentration of uric acid and its potential clinical implications]. Aten Primaria 2000;26:468–71.
18. Galman C, Angelin B, Rudling M. Bile acid synthesis in humans has a rapid diurnal variation that is asynchronous with cholesterol synthesis. Gastroenterology 2005;129:1445–53.

Published OnlineFirst March 3, 2016; DOI: 10.1158/1055-9965.EPI-15-1206

Cancer Epidemiology, Biomarkers & Prevention

AACR American Association for Cancer Research

Impact of Pre-analytic Blood Sample Collection Factors on Metabolomics

Mary K. Townsend, Ying Bao, Elizabeth M. Poole, et al.

Cancer Epidemiol Biomarkers Prev 2016;25:823-829. Published OnlineFirst March 3, 2016.